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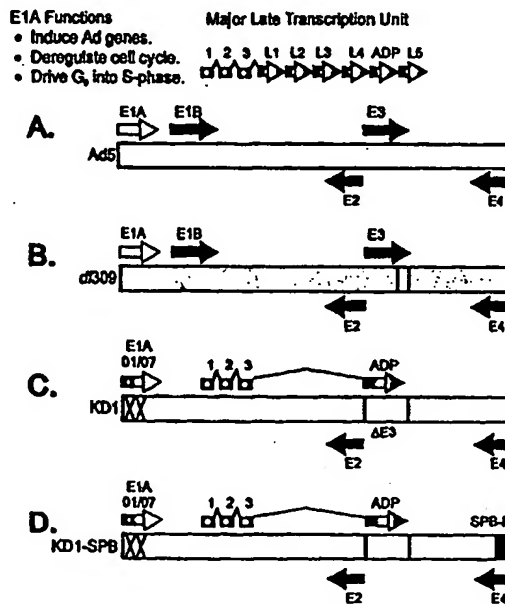
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(75) Inventors/Applicants (for US only): WOLD, William S., M. [CA/US]; 1609 Adgers Wharf Boulevard, Chesterfield, MO 63017 (US). TOTH, Karoly [HU/US]; 7345 Fernbrook, Apt. 202, St. Louis, MO 63123 (US). DORONIN, Konstantin [RU/US]; 8133 Briarhaven Trail, Apt. 304, St. Louis, MO 63123 (US). TOLLEFSON, Ann, E. [US/US]; 9026 Philo Avenue, St. Louis, MO 63123 (US).
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(54) Title: REPLICATION-COMPETENT ANTI-CANCER VECTORS



(57) Abstract: Novel vectors which are replication-competent in neoplastic cells and which overexpress an adenovirus death protein are disclosed. Some of the disclosed vectors are replication-restricted to neoplastic cells or to neoplastic alveolar type II cells. Compositions and methods for promoting the death of neoplastic cells using these replication-competent vectors are also disclosed.

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### Replication-Competent Anti-Cancer Vectors

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#### 5 Background of the Invention

##### (1) Field of the Invention

This invention relates generally to the treatment of cancer and more particularly to vectors which replicate in neoplastic cells and which overexpress an adenovirus death protein (ADP) and to the use of these vectors in treating human cancer.

#### 10 (2) Description of the Related Art

Cancer is a leading cause of death in the United States and elsewhere. Depending on the type of cancer, it is typically treated with surgery, chemotherapy, and/or radiation. These treatments often fail: surgery may not remove all the cancer; some cancers are resistant to chemotherapy and radiation therapy; and chemotherapy-resistant tumors frequently develop.

15 New therapies are necessary, to be used alone or in combination with classical techniques.

One potential therapy under active investigation is treating tumors with recombinant viral vectors expressing anti-cancer therapeutic proteins. Adenovirus-based vectors contain several characteristics that make them conceptually appealing for use in treating cancer, as well as for therapy of genetic disorders. Adenoviruses (hereinafter used interchangeably with

"Ads") can easily be grown in culture to high titer stocks that are stable. They have a broad host range, replicating in most human cancer cell types. Their genome can be manipulated by site-directed mutation and insertion of foreign genes expressed from foreign promoters.

The adenovirion consists of a DNA-protein core within a protein capsid (reviewed by Stewart et al., "Adenovirus structure by x-ray crystallography and electron microscopy." in: *The Molecular Repertoire of Adenoviruses*, Doerfler, W. et al., (ed.), Springer-Verlag, Heidelberg, Germany, p. 25-38). Virions bind to a specific cellular receptor, are endocytosed, and the genome is extruded from endosomes and transported to the nucleus. The genome is a linear duplex DNA of about 36 kbp, encoding about 36 genes (Fig. 1A). In the nucleus, the "immediate early" E1A proteins are expressed initially, and these proteins induce expression of the "delayed early" proteins encoded by the E1B, E2, E3, and E4 transcription units (reviewed by Shenk, T. "Adenoviridae: the viruses and their replication" in: *Fields Virology*, Field, B.N. et al., Lippencott-Raven, Philadelphia, p. 2111-2148). E1A proteins also induce or repress cellular genes, resulting in stimulation of the cell cycle. About 23 early proteins function to usurp the cell and initiate viral DNA replication. Viral DNA replicates at about 7 h post-infection (p.i.), then late genes are expressed from the "major late" transcription unit. Major late mRNAs are synthesized from the common "major late promoter" by alternative pre-mRNA processing. Each late mRNA contains a common "tripartite leader" at its 5'-terminus (exons 1, 2, and 3 in Fig. 1), which allows for efficient translation of Ad late mRNAs. Cellular protein synthesis is shut off, and the cell becomes a factory for making viral proteins. Virions assemble in the nucleus at about 1 day p.i., and after 2-3 days the cell lyses and releases progeny virus. Cell lysis is mediated by the E3 11.6K protein, which has been renamed "adenovirus death protein" (ADP) (Tollefson et al., *J. Virol.* 70:2296-2306, 1996; Tollefson et al., *Virol.* 220:152-162, 1996). The term ADP as used herein in a generic sense refers collectively to ADP's from adenoviruses such as, e.g. Ad type 1 (Ad1), Ad type 2 (Ad2), Ad type 5 (Ad5) or Ad type 6 (Ad6) all of which express homologous ADP's with a high degree of sequence similarity.

Human adenovirus type 5 (Ad5) is particularly useful for cancer gene therapy. It primarily causes asymptomatic or mild respiratory infections in young children, followed by long term effective immunity. Fatalities are extremely rare except when the patient is immunocompromised (Horwitz, M. S., Adenoviruses, p. 2149-2171 In B. N. Fields, D. M. Knipe, and P. M. Howley (eds.), *Fields Virology*, Lippincott-Raven Publishers, Philadelphia, PA, 1996). Ad5 is very well understood, can be grown in culture to high titer stocks that are stable, and can replicate in most human cancer cell types (Shenk, T., Adenoviridae: the viruses and their replication, p. 2111-2148. In B. N. Fields, D. M. Knipe, and P. M. Howley



(eds.), Fields Virology, Lippincott-Raven, Philadelphia, 1996). Its genome can be manipulated by site-directed mutagenesis and insertion of foreign sequences.

The Ad vectors being investigated for use in anti-cancer and gene therapy are based on recombinant Ad's that are either replication-defective or replication-competent. Typical  
5 replication-defective Ad vectors lack the E1A and E1B genes (collectively known as E1) and contain in their place an expression cassette consisting of a promoter and pre-mRNA processing signals which drive expression of a foreign gene. The E1A proteins induce transcription of other Ad genes, and in nontransformed cells they deregulate the cell cycle, induce or repress a variety of cellular genes, and force cells from G<sub>0</sub> into S-phase 48 (White,  
10 E., *Semin. Virol.* 8:505-513, 1998; Wold et al., pp. 200-232 *In* A.J. Cann (ed.), *DNA Virus Replication: Frontiers in Molecular Biology*, Oxford University Press, Oxford). The E1B proteins inhibit cellular apoptosis. *Id.* These vectors are unable to replicate because they lack the E1A genes required to induce Ad gene expression and DNA replication. In addition, the E3 genes are usually deleted because they are not essential for virus replication in cultured  
15 cells.

A number of investigators have constructed replication-defective Ad vectors expressing anti-cancer therapeutic proteins. Usually, these vectors have been tested by direct injection of human tumors growing in mouse models. Most commonly, these vectors express the thymidine kinase gene from herpes simplex virus, and the mice are treated with  
20 gancyclovir to kill cells transduced by the vector (see e.g., Felzmann et al., *Gene Ther.* 4:1322-1329, 1997). Another suicide gene therapy approach involves injecting tumors with a replication defective Ad vector expressing cytosine deaminase, followed by administration of 5-fluorocytosine (Topf et al., *Gene Ther.* 5:507-513, 1998). Investigators have also prepared and tested replication-defective Ad vectors expressing a cytokine-such as IL-2, IL-12, IL-6,  
25 tumor necrosis factor (TNF), type I interferons, or the co-stimulatory molecule B7-1 in the anticipation that the Ad-expressed cytokine will stimulate an immune response, including cytotoxic T-lymphocytes (CTL), against the tumor (Felzmann et al., *supra*; Putzer et al., *Proc. Natl. Acad. Sci. USA* 94:10889-10894, 1997). Other vectors express tumor antigens (e.g. melanoma MART1), proteins that de-regulate the cell cycle and induce apoptosis (p53, pRB, p21<sup>Kip1/WAF1</sup>, p16<sup>CDKN2</sup>, and even Ad E1A), and ribozymes. An Ad vector expressing FasL  
30 induces apoptosis and tumor regression of a mouse tumor (Arai et al., *Proc. Natl. Acad. Sci. USA* 94:13862-13867, 1997).

Despite these generally positive reports, it is recognized in the art that replication-defective Ad vectors have several characteristics that make them suboptimal for  
35 use in therapy. For example, production of replication-defective vectors requires that they be grown on a complementing cell line that provides the E1A proteins in trans. Such cell lines

are fastidious, and generation of virus stocks is time-consuming and expensive. In addition, although many foreign proteins have been expressed from such vectors, the level of expression is low compared to Ad late proteins.

To address these problems, several groups have proposed using replication-competent Ad vectors for therapeutic use. Replication-competent vectors retain Ad genes essential for replication and thus do not require complementing cell lines to replicate. Replication-competent Ad vectors lyse cells as a natural part of the life cycle of the vector. Another advantage of replication-competent Ad vectors occurs when the vector is engineered to encode and express a foreign protein. Such vectors would be expected to greatly amplify synthesis of the encoded protein *in vivo* as the vector replicates. However, in order to prevent RC vectors from damaging normal tissues and causing disseminated viremia, it is important that they have some feature that limits their replication to cancer cells.

Wyeth Laboratories developed replication-competent Ad vectors for vaccination purposes, using vaccine strains of Ad serotypes 4, 7, and 5 (Lubeck et al., *AIDS Res. Hum. Retroviruses* 10:1443-1449, 1994). Foreign genes were inserted into the E3 region (with the E3 genes deleted) or into a site at the right end of the genome. Two foreign genes used were hepatitis B surface antigen and the HIV envelope protein. They obtained good expression in culture, and were able to raise antisera in animal models. Phase I human trials were ambiguous, and the project was mostly abandoned.

Onyx Pharmaceuticals recently reported on adenovirus-based anti-cancer vectors which are replication deficient in non-neoplastic cells but which exhibit a replication phenotype in neoplastic cells lacking functional p53 and/or retinoblastoma (pRB) tumor suppressor proteins (U.S. Patent No. 5,677,178; Heise et al., *Nature Med.* 6:639-645, 1997; Bischoff et al., *Science* 274:373-376, 1996). This phenotype is reportedly accomplished by using recombinant adenoviruses containing a mutation in the E1B region that make the encoded E1B-55K protein incapable of binding to p53 and/or a mutation(s) in the E1A region which make the encoded E1A protein (p289R or p243R) incapable of binding to pRB and/or the cellular 300 kD polypeptide and/or the 107 kD polypeptide. E1B-55K has at least two independent functions: it binds and inactivates the tumor suppressor protein p53, and it is required for efficient transport of Ad mRNA from the nucleus. Because these E1B and E1A viral proteins are involved in forcing cells into S-phase, which is required for replication of adenovirus DNA, and because the p53 and pRB proteins block cell cycle progression, the recombinant adenovirus vectors described by Onyx should replicate in cells defective in p53 and/or pRB, which is the case for many cancer cells, but not in cells with wild-type p53 and/or pRB. Onyx has reported that replication of an adenovirus lacking E1B-55K, which is named ONYX-015, was restricted to p53-minus cancer cell lines (Bischoff et al., *supra*), and

that ONYX-015 slowed the growth or caused regression of a p53-minus human tumor growing in nude mice (Heise et al., *supra*). Others have challenged the Onyx report claiming that replication of ONYX-015 is independent of p53 genotype and occurs efficiently in some primary cultured human cells (Harada and Berk, *J. Virol* 73:5333-5344, 1999). It is now  
5 known that ONYX-015 can replicate in cells with wild-type p53 (Goodrum et al., *J. Virol.* 72:9479-9490, 1998; Harada et al., *J. Virol.* 73:5333-5344, 1999; Hay et al., *Hum. Gene Ther.* 10:579-590, 1999; Rothmann et al., *J. Virol.* 72:9470-9478, 1998; Turnell et al., *J. Virol.* 73:2074-2083, 1999). ONYX-015 does not replicate as well as wild-type adenovirus because E1B-55K is not available to facilitate viral mRNA transport from the nucleus. Also, ONYX-  
10 015 expresses less ADP than wild-type virus (see Example 1 below).

As an extension of the ONYX-015 concept, a replication-competent adenovirus vector was designed that has the gene for E1B-55K replaced with the herpes simplex virus thymidine kinase gene (Wilder et al., *Gene Therapy* 6:57-62, 1999). The group that constructed this vector reported that the combination of the vector plus gancyclovir showed a  
15 therapeutic effect on a human colon cancer in a nude mouse model (Wilder et al., *Cancer Res.* 59:410-413, 1999). However, this vector lacks the gene for ADP, and accordingly, the vector will lyse cells and spread from cell-to-cell less efficiently than an equivalent vector that expresses ADP. The gene for ADP is also lacking in another replication-competent adenovirus vector that has been described, in which a minimal enhancer/promoter of the  
20 human prostate specific antigen was inserted into the adenovirus E1A enhancer/promoter (Rodriguez et al., *Cancer Res.* 57:2559-2563, 1997).

Another strategy for replication-competent vector improvement is to place replication under the control of tissue-specific promoters. One group replaced the basal E1A promoter with a modified promoter for  $\alpha$ -fetoprotein (AFP) (Hallenbeck et al., *Hum. Gene Ther.*  
25 10:1721-1733, 1999). AFP is expressed in the liver during development, but it is not expressed in adults. However, it is expressed in 70-80% of patients with hepatocellular carcinoma. Growth of this vector was limited to AFP-expressing cells and the vector showed some suppression of xenotransplants. *Id.* A series of RC vectors has also been developed that have expression of the E1A and E1B genes dependent on the prostate tumor-specific  
30 prostate specific antigen (PSA) and kallikrein promoters/enhancers (Rodriguez et al., *Cancer Res.* 60:1196, 1997; Yu et al., *Cancer Res.* 59:4200-4203, 2000; Yu et al., *Cancer Res.* 59:1498-1504, 1999).

Thus, there is a continuing need for vectors that replicate and spread efficiently in tumors but that can be modified such that they replicate poorly or not at all in normal tissue.

35 Summary of the Invention

Briefly, therefore, the present invention is directed to novel vectors which are replication competent in neoplastic cells and which overexpress an adenovirus death protein (ADP). The work reported herein demonstrates the discovery that overexpression of ADP by a recombinant adenovirus allows the construction of a replication-competent adenovirus that kills neoplastic cells and spreads from cell-to-cell at a rate similar to or faster than that exhibited by adenoviruses expressing wild-type levels of ADP, even when the recombinant adenovirus contains a mutation that would otherwise reduce its replication rate in non-neoplastic cells. This discovery was unexpected because it could not have been predicted from what was known about adenovirus biology that Ad vectors overexpressing ADP remain viable and that the infected cells are not killed by the higher amounts of ADP before the Ad vector produces new virus particles that can spread to other tumor cells. Indeed, naturally-occurring adenoviruses express ADP in low amounts from the E3 promoter at early stages of infection, and begin to make ADP in large amounts only at 24-30 h p.i., once virions have been assembled in the cell nucleus. It is believed that other non-adenoviral vectors can be used to deliver ADP's cell-killing activity to neoplastic cells, including other viral vectors and plasmid expression vectors.

Thus, in one preferred embodiment, the ADP-expressing vector comprises a recombinant adenovirus lacking expression of at least one E3 protein selected from the group consisting of: gp19K; RID $\alpha$  (also known as 10.4K); RID $\beta$  (also known as 14.5K) and 14.7K. Because these E3 proteins inhibit immune-mediated inflammation and/or apoptosis of Ad-infected cells, it is believed that a recombinant adenovirus lacking one or more of these E3 proteins will stimulate infiltration of inflammatory and immune cells into a tumor treated with the adenovirus and that this host immune response will aid in destruction of the tumor as well as tumors that have metastasized. The ADP expressed by preferred embodiments comprises a naturally-occurring amino acid sequence from a human adenovirus of subgroup C, namely Ad1, Ad2, Ad5 and Ad6.

In another embodiment, replication of the vector is restricted to neoplastic cells. Such replication-restricted vectors are useful in treating cancer patients in which it is desirable to eliminate or reduce damage to normal cells and tissues that might be caused by the vector, particularly viral vectors that kill the host cell as part of their life cycle. In preferred embodiments, a recombinant adenovirus has a replication-restricted phenotype because the recombinant adenovirus is incapable of expressing an E1A viral protein which binds the pRB and the p300/CBP proteins or because the E4 promoter has been substituted with a promoter that is activated only in neoplastic cells and/or cells of a specific tissue.

In yet another embodiment, the invention provides a vector which overexpresses ADP and whose replication is under the control of a tissue specific promoter, tumor specific

promoter or an inducible promoter. In preferred embodiments, the vector comprises a recombinant adenovirus in which the tissue specific promoter or inducible promoter is substituted for the E4 promoter. Such vectors are useful for restricting replication of the vector and its ADP-mediated cell killing to cells of a particular type or to cells exposed to an exogenous agent that activates the promoter. A preferred tissue-specific or inducible vector also expresses a phenotype that restricts its replication to neoplastic cells.

In yet another embodiment, the invention provides a vector which overexpresses ADP but which is not restricted to tumors by a specific genetic modification. Such a vector is more destructive to neoplastic cells than even the naturally occurring Ad's of subgroup C. In preferred embodiments, this vector could be used for patients with terminal cancer not treatable by another method, and who have pre-existing neutralizing antibodies to Ad or to which neutralizing antibodies can be administered.

In still another embodiment, the invention provides a composition comprising a first recombinant virus which is replication competent in a neoplastic cell and overexpresses the adenovirus death protein. In one embodiment, the recombinant virus is contained within a delivery vehicle comprising a targeting moiety that limits delivery of the virus to cells of a certain type. With this embodiment, the replication-competent vector can be of any ADP-overexpressing configuration described herein. In some embodiments, the composition also comprises a second recombinant virus which is replication-defective and which expresses an anti-cancer gene product. In some embodiments, the replication-defective vector may be engineered to overexpress ADP when replication of this vector is complemented by a replication-competent vector. The recombinant virus complements spread of the replication-defective virus, as well as its encoded anti-cancer product, throughout a tumor. In preferred embodiments, the first recombinant virus is a recombinant adenovirus whose replication is restricted to neoplastic cells and/or which lacks expression of one or more of the E3 gp19K; RID $\alpha$ ; RID $\beta$ ; and 14.7K proteins.

In additional embodiments, the invention provides replication-competent vectors that overexpresses an ADP and also expresses an anti-cancer product. As with previous embodiments, the vector can be of any ADP-overexpressing configuration provided herein. Preferably, replication of the virus is engineered to (a) be restricted to neoplastic cells, e.g., by replacing the E4 promoter with a tissue specific or tumor specific promoter and/or (b) lack expression of one or more of the E3 gp19K; RID $\alpha$ ; RID $\beta$ ; and 14.7K proteins. In some embodiments, the anti-cancer product is inserted into the E3 region.

The ADP-expressing vectors and compositions of the invention are useful in a method for promoting death of a neoplastic cell. The method comprises contacting the neoplastic cell with a vector which is replication-competent in the neoplastic cell and which

overexpresses ADP. Where the neoplastic cell comprises a tumor in a patient, the vector is administered directly to the tumor or, in other embodiments, the vector is administered to the patient systemically or in a delivery vehicle containing a targeting moiety that directs delivery of the vector to the tumor. In embodiments where the vector is a recombinant virus, the method can also comprise passively immunizing the patient against the virus.

In yet another embodiment of the invention, the vector may be used in combination with radiation therapy. The radiation therapy can be any form of radiation therapy used in the art such as for example, external beam radiation such as x-ray treatment, radiation delivered by insertion of radioactive materials within the body near or at the tumor site such as treatment with gamma ray emitting radionuclides, particle beam therapy which utilizes neutrons or charged particles and the like. In addition, this embodiment encompasses the use of more than one of the vectors of the present invention in a cocktail in combination with radiation therapy.

Another embodiment of the invention involves the use of the recombinant vector in combination with chemotherapy as has been disclosed for other adenovirus vectors (U.S. Patent No. 5,846,945). Chemotherapeutic agents are known in the art and include antimetabolites including pyrimidine-analogue and purine-analogue antimetabolites, plant alkaloids, antitumor antibiotics, alkylating agents and the like. The use of more than one of the vectors of the present invention with a chemotherapeutic agent or agents is also contemplated within this embodiment.

Among the several advantages found to be achieved by the present invention, therefore, may be noted the provision of replication-competent vectors, particularly viruses, which rapidly kill cancer cells and spread from cell-to-cell in a tumor; the provision of such vectors whose replication can be induced or which is restricted to tumors and/or to cells of a certain tissue type; and the provision of compositions and methods for anti-cancer therapy which cause little to no side effects in normal tissues.

#### Brief Description of the Drawings

Figure 1 is a schematic of gene expression in Ad5 (Fig. 1A) and KD3, a preferred embodiment of the invention (Fig. 1B), in which the respective genomes are represented by the stippled bars and transcription units represented by arrows above and below the bars, with the E3 proteins listed above the arrows for the E3 transcription unit, and the L1 to L5 families of late mRNA's indicated.

Figure 2 illustrates the overexpression of ADP by KD1, KD3, GZ1, and GZ3 showing an immunoblot of proteins isolated from human A549 cells infected with the indicated viruses and probed with an anti-ADP antibody, with ADP indicating differently glycosylated and proteolytically processed forms of ADP.

Figure 3 illustrates that the E1A *dl1101/1107* mutation referred to in the figure and hereinafter as *dl01/07*, retards expression of late proteins, showing an immunoblot of E1A proteins and late proteins in A549 cells infected with the indicated viruses in the absence (Figs. 3A and 3B) or presence (Figs. 3C and 3D) of *dl327*, which has a wild-type E1A region and has a deletion of all E3 genes but the gene encoding the 12.5K protein (Figs. 3C and 3D). An antiserum specific to the E1A proteins was used for Fig. 3A and 3C. An antiserum raised against Ad5 virions was used for Figs. 3B and 3D.

Figure 4 illustrates that KD1 and KD3 kill cells more efficiently than control viruses that express less or no ADP, showing a graph of the percent of A549 cells infected with the indicated viruses that were viable at the indicated days p.i. as determined by trypan blue exclusion.

Figure 5 is a cell spread assay illustrating that overexpression of ADP enhances spread of virus from cell to cell, showing monolayers infected with the indicated viruses at the indicated PFU/cell which were treated at 7 days p.i. with crystal violet, which stains live cells but not dead cells.

Figure 6 illustrates that KD1 and KD3 replicate well in growing cells but not in growth-arrested cells showing the virus titer extracted from growing or growth arrested HEL-229 cells at various times following infection with 100 PFU/ml of the following viruses: *dl309* (Fig. 6A), *dl01/07* (fig. 6B), KD1 (Fig. 6C) and KD3 (Fig 6D).

Figure 7 illustrates that KD1 and KD3 are defective in killing primary human bronchial epithelial cells showing these cell monolayers infected at 30% confluency with 10 PFU/ml of the indicated viruses and stained at 5 days p.i. with neutral red.

Figure 8 illustrates that KD1 and KD3 reduce the growth rate of human A549 cell tumors growing in nude mice, showing in Fig. 8A a graph of average-fold increase in tumor size plotted against the number of weeks following infection of the tumor with buffer or with  $5 \times 10^7$  PFU at weekly intervals of or the indicated viruses, and showing in Fig. 8B a similar graph of tumors injected once with  $5 \times 10^8$  PFU of KD3 or GZ3.

Figure 9 illustrates that KD1 and KD3 reduce the growth rate of human Hep3B cell tumors growing in nude mice, showing a graph of average-fold increase in tumor size plotted against the number of weeks following injection of the tumor with buffer or with  $5 \times 10^7$  PFU of *dl309*, KD1 or KD3 at twice weekly intervals of the indicated viruses.

Figure 10 illustrates that KD1 and KD3 complement the replication and spread of Ad- $\beta$ -gal, a replication-defective vector that expresses  $\beta$ -galactosidase, using an infectious center assay showing in Fig. 10A a picture of A549 cell monolayers seeded with A549 cells infected with Ad- $\beta$ -gal alone or with the indicated viruses, with Figs 10B and 10C showing close-up views of two of the monolayers of Fig. 10A.

Figure 11 is a bar graph illustrating that KD1 and KD3 increase the expression of luciferase in human Hep3B cell tumors growing in nude mice, using an assay in which tumors were injected with the indicated combinations of viruses, then were extracted 2 weeks p.i. and assayed for luciferase activity. The numbers in parentheses indicated the fold increase in luciferase activity compared to that of the Adluc vector plus buffer.

Figure 12 is a graph showing the results of a standard plaque development assay for KD1 and KD1-SPB on A549 cells engineered to express the TTF1 transcription factor (A549/TTF1) and the parental 549 cells, in which data are plotted as the number of plaques observed on a particular day in the assay divided by the final number of plaques observed for that virus multiplied by 100.

Figure 13 is a cell spread assay for KD1 and KD1-SPB on H441 cells and Hep3B cells, where cells were infected with the indicated amounts of KD1 or KD1-SPB and H441 cells and Hep3B cells were stained with crystal violet at 5 days p.i. and 8 days p.i., respectively.

Figure 14 is a graph showing the results of a standard plaque development assay for d1309 and two preferred embodiments of the invention, GZ1 and GZ3, in which data are plotted as the number of plaques observed on a particular day in the assay divided by the final number of plaques observed for that virus multiplied by 100.

Figure 15 is a cell spread assay illustrating that the combination of KD1, KD3, GZ1, or GZ3 with x-ray radiation is more effective in destroying A549 cell monolayers than is virus vector alone or radiation alone, wherein cells were infected with the indicated amounts of the indicated viruses, radiated with 600 centigreys (cGy) of x-radiation (bottom panel), or mock radiated (top panel), then stained with crystal violet at 6 days p.i.

Figure 16 is a graph of a cell spread assay illustrating that  $10^3$  PFU of KD1, KD3, GZ1, or GZ3 used in combination with 150, 300, or 600 centigreys of radiation is more effective in destroying A549 cell monolayers than virus vector alone or radiation alone. Cell viability is based on the amount of crystal violet extracted from the culture wells, using the mock-infected non-radiated well as 100% viability.

Figure 17 illustrates that the combination of KD3 or GZ3 plus x-ray radiation is more effective in reducing the growth of A549 cell tumors growing in nude mice than KD3 alone or GZ3 alone.

Figure 18 illustrates a structure-function analysis of ADP, showing in Fig. 18A the amino acid sequence of the adenovirus death protein encoded by Ad2, with the various putative domains and glycosylation sites labeled and showing in Fig. 18B a schematic of the ADP gene in *rec700* and in the indicated deletion mutants, with the right column



summarizing the death promoting phenotype of the various mutants as a percentage of the wild-type phenotype.

Figures 19A and 19B illustrate a cell viability assay of the indicated ADP mutants showing a graph of viability as determined by trypan blue exclusion plotted against hours (Fig. 19A) or days (Fig. 19B) postinfection.

Figure 20 depicts the amino acid sequence, shown in single letter code, for the ADP proteins of Ad1, Ad2, Ad5, and Ad6 (SEQ ID NOS:5-8), for the Ad2 ADP mutants *dl716*, *dl715*, *dl714*, and *dl737* (SEQ ID NOS:9-12), and for the putative luminal domain (SEQ ID NO:17), the transmembrane domain (SEQ ID NO:18), the cytosolic basic-proline domain (SEQ ID NO:19), and the remainder of the cytosolic domain (SEQ ID NO:20) of the ADP protein of Ad2.

Figure 21 presents the complete nucleotide sequence of the genome of Ad5.

Figure 22 presents the complete nucleotide sequence of the genome of KD1 (SEQ ID NO:1).

Figure 23 presents the complete nucleotide sequence of the genome of KD3 (SEQ ID NO:2).

Figure 24 is a schematic of the following vectors: A. Ad5. The stippled bar indicates the DNA genome of 36 kbp. The open arrow indicates the immediate early E1A transcription unit, and the black arrows are the delayed early E1B, E2, E3, and E4 transcription units. The hatched arrows indicate the five families of major late mRNAs, and also the ADP mRNA, which is synthesized as part of the major late transcription unit. Each major late mRNA has a tripartite leader (leaders 1, 2, and 3) spliced to its 5' terminus. B. dl309. dl309 is identical to Ad5 except it has the E3-RID and E3-14.7K genes deleted. dl309 expresses ADP at levels similar to Ad5. C. KD1. KD1 has two small deletions (indicated by "X" marks) in the E1A gene that abolish binding of the E1A proteins to pRB or p300/CBP. It lacks all E3 genes except adp. ADP is expressed earlier in infection and in greater abundance than is ADP from Ad5 or dl309 Doronin et al., *J. Virol.* 74:6147-6155. D. KD1-SPB. KD1-SPB is identical to KD1, except it has the E4 promoter replaced by the promoter for Surfactant Protein B (SPB-P).

Figure 25 presents graphs illustrating that KD1-SPB grows as well as KD1 in H441 lung carcinoma cells but much more poorly than KD1 in Hep 3B hepatoma cells. CsCl-banded stocks of KD1-SPB and KD1 were titrated using standard methods (Tollefson et al., p. 1-9 In W.S.M. Wold (ed.), *Adenovirus Methods and Protocols*. Humana Press, Inc., Totowa, NJ, 1998) on 293-E4 or 293 cells (A), or on A549 cells (B). The data are plotted as the number of plaques seen on any day of the plaque assay as a percentage of the number of plaques seen on the final day of the assay (Tollefson et al., *Virology* 220:152-162, 1996).

Figure 26 presents micrographs illustrating that KD1-SPB induces CPE in H441 cells but not Hep 3B cells. H441 and Hep 3B monolayers were mock-infected or infected with 10 PFU/cell of KD1 or KD1-SPB, then photographed under phase contrast at 4 or 7 days p.i.

Figure 27 depicts Southern hybridizations and a graph illustrating that KD1-SPB DNA is synthesized efficiently in H441 but not Hep 3B cells. H441 or Hep 3B cells were infected with 10 PFU/cell of KD1 or KD1-SPB. Total genomic DNA was isolated at 0, 5, 24, 48, 72, and 96 h p.i., digested with HindIII, resolved by agarose gel electrophoresis, blotted, and hybridized with <sup>32</sup>P-labeled Ad DNA. A. Autoradiogram. B. PhosphorImager quantitation of the DNA bands in Panel A.

Figure 28 presents graphs depicting single step growth curves showing that KD1-SPB grows well in H441 but not Hep 3B cells. Cells were infected with 10 PFU/cell of KD1 or KD1-SPB. Vectors were extracted at the indicated days p.i. and titers determined by plaque assay.

Figure 29 depicts immunoblots showing that KD1-SPB expresses E4ORF3 and ADP in H441 but not Hep 3B cells. Cells were infected with 10 PFU/cell of KD1 or KD1-SPB. At 24 h p.i., protein extracts were analyzed for E1A, E4ORF3, and ADP using specific antisera. The E1A proteins appear as multiple bands. ADP appears as two bands; the upper band is glycosylated and the lower band is a proteolytically cleaved species (Scaria et al., *Virology* 191:743-753, 1992; Tollefson et al., *J. Virol.* 66:3633-3642).

Figure 30 depicts immunofluorescence micrographs showing that KD1-SPB expresses E4ORF3 in H441 but not Hep 3B cells. Cells growing on coverslips were infected with 20 PFU/cell of KD1, KD1-SPB, or dl309 (wild-type). At 48 h (Panel A) or 6 days (Panel B), cells were fixed and stained with a rabbit polyclonal antipeptide antiserum against E4ORF3. Photographs were taken using a 100X Planapo lens. Each panel shows about 8 nuclei. This figure is part of the same experiment shown in Figure 31.

Figure 31 depicts immunofluorescence micrographs showing that KD1-SPB does not express E2-DBP or fiber efficiently in Hep 3B cells. Hep 3B cells were infected with 20 PFU/cell of KD1-SPB or KD1. At 48 h (A) or 6 days (B) p.i., cells were fixed and double-stained using a rabbit polyclonal antiserum against DBP and a mouse monoclonal antibody against fiber. The same fields are shown for DBP and fiber. This figure is part of the same experiment shown in Figure 30.

Figure 32 presents graphs illustrating that KD1-SPB lyses H441 but not Hep 3B as efficiently as KD1. H441 or Hep 3B cells were mock-infected or infected with 20 PFU/cell of KD1 or KD1-SPB. Cell lysis was determined by release of lactate dehydrogenase from the cells into the medium.

Figure 33 presents graphs illustrating that KD1-SPB suppresses growth of H441 tumors in nude mice equally as well as KD1. Tumor cells were injected into flanks of nude mice and allowed to grow to about 100  $\mu$ l (H441) or 150  $\mu$ l (Hep 3B) volumes. Tumors (n = 10) were injected with DMEM (mock) or with  $5 \times 10^7$  PFU of KD1 or KD1-SPB. Injections of the viruses were repeated twice weekly for 3 weeks to a total dose of  $3.0 \times 10^8$  PFU per tumor. Tumors were measured and the mean fold-increase in tumor size was calculated.

#### Description of the Preferred Embodiments

In accordance with the present invention, it has been discovered that overexpression of ADP by a recombinant adenovirus results in faster lysis of cells and spread of the virus throughout a cell monolayer than viruses expressing wild-type levels of ADP. It has also been discovered that this function for ADP is manifest in an adenovirus that contains E1A mutations that restrict adenoviral replication to neoplastic cells. Thus, vectors which are both replication competent in neoplastic cells and which overexpress ADP should be useful in anti-cancer therapy.

In the context of this disclosure, the following terms will be defined as follows unless otherwise indicated:

"Naturally-occurring" as applied to an object such as a polynucleotide, polypeptide, or virus means that the object can be isolated from a source in nature and has not been intentionally modified by a human.

"Neoplastic cell" means a cell which exhibits an aberrant growth phenotype characterized by a significant loss of control of cell proliferation and includes actively replicating cells as well as cells in a temporary non-replicative resting state ( $G_1$  or  $G_2$ ). A neoplastic cell may have a well-differentiated phenotype or a poorly-differentiated phenotype and may comprise a benign neoplasm or a malignant neoplasm.

"Recombinant virus" means any viral genome or virion that is different than a wild-type virus due to a deletion, insertion, or substitution of one or more nucleotides in the wild-type viral genome. The recombinant virus can have changes in the number of amino acid sequences encoded and expressed or in the amount or activity of proteins expressed by the virus. In particular, the term includes recombinant viruses generated by the intervention of a human.

"Replication-competent" as applied to a vector means that the vector is capable of replicating in normal and/or neoplastic cells. As applied to a recombinant virus, "replication-competent" means that the virus exhibits the following phenotypic characteristics in normal and/or neoplastic cells: cell infection; replication of the viral genome; and production and release of new virus particles; although one or more of these characteristics need not occur at the same rate as they occur in the same cell type infected by a wild-type virus, and may occur

at a faster or slower rate. Where the recombinant virus is derived from a virus such as adenovirus that lyses the cell as part of its life cycle, it is preferred that at least 5 to 25% of the cells in a cell culture monolayer are dead 5 days after infection. Preferably, a replication-competent virus infects and lyses at least 25 to 50%, more preferably at least 75%, and most preferably at least 90% of the cells of the monolayer by 5 days post infection (p.i.).

"Replication-defective" as applied to a recombinant virus means the virus is incapable of, or is greatly compromised in, replicating its genome in any cell type in the absence of a complementing replication-competent virus. Exceptions to this are cell lines such as 293 cells that have been engineered to express adenovirus E1A and E1B proteins.

"Replication-restricted" as applied to a vector of the invention means the vector replicates better in a dividing cell, i.e. either a neoplastic cell or a non-neoplastic, dividing cell, than in a cell of the same type that is not neoplastic and/or not dividing, which is also referenced herein as a normal, non-dividing cell. Preferably, a replication-restricted virus kills at least 10% more neoplastic cells than normal, non-dividing cells in cell culture monolayers of the same size, as measured by the number of cells showing cytopathic effects (CPE) at 5 days p.i. More preferably, between 25% and 50%, and even more preferably, between 50% and 75% more neoplastic than normal cells are killed by a replication-restricted virus. Most preferably, a replication-restricted adenovirus kills between 75% and 100% more neoplastic than normal cells in equal sized monolayers by 5 days p.i.

In one embodiment the invention provides a vector that is replication-competent in neoplastic cells and which overexpresses an ADP. Vectors useful in the invention include but are not limited to plasmid-expression vectors, bacterial vectors such as *Salmonella* species that are able to invade and survive in a number of different cell types, vectors derived from DNA viruses such as human and non-human adenoviruses, adenovirus associated viruses (AAVs), poxviruses, herpesviruses, and vectors derived from RNA viruses such as retroviruses and alphaviruses. Preferred vectors include recombinant viruses engineered to overexpress an ADP. Recombinant adenoviruses are particularly preferred for use as the vector, especially vectors derived from Ad1, Ad2, Ad5 or Ad6.

Vectors according to the invention overexpress ADP. As applied to recombinant Ad and AAV vectors, the term "overexpresses ADP" means that more ADP molecules are made per viral genome present in a dividing cell infected by the vector than expressed by any previously known recombinant adenoviral vector or AAV in a dividing cell of the same type. As applied to other, non-adenoviral vectors, "overexpresses ADP" means that the virus expresses sufficient ADP to lyse a cell containing the vector.

Vectors overexpressing ADP can be prepared using routine methodology. See, e.g., *A Laboratory Cloning Manual*, 2nd Ed., vol. 3, Sambrook et al., eds., Cold Spring Harbor

Laboratory Press, 1989. For example, a polynucleotide encoding the ADP can be cloned into a plasmid expression vector known to efficiently express heterologous proteins in mammalian cells. The polynucleotide should also include appropriate termination and polyadenylation signals. Enhancer elements may also be added to the plasmid to increase the amount of ADP expression. Viral vectors overexpressing ADP can be prepared using similar materials and techniques.

Where the virus is a recombinant adenovirus, overexpression of ADP can be achieved in a multitude of ways. In general, any type of deletion in the E3 region that removes a splice site for any of the E3 mRNAs will lead to overexpression of the mRNA for ADP, inasmuch as more of the E3 pre-mRNA molecules will be processed into the mRNA for ADP. This is exemplified in the KD1, KD3, GZ1 and GZ3 vectors (SEQ ID NOS:1-4) whose construction is described below. Other means of achieving overexpression of ADP in Ad vectors include, but are not limited to: insertion of pre-mRNA splicing and cleavage/polyadenylation signals at sites flanking the gene for ADP; expression of ADP from another promoter, e.g. the human cytomegalovirus promoter, inserted into a variety of sites in the Ad genome; and insertion of the gene for ADP behind the gene for another Ad mRNA, together with a sequence on the 5' side of the ADP sequence that allows for internal initiation of translation of ADP, e.g. the Ad tripartite leader or a viral internal ribosome initiation sequence.

The ADP expressed by a vector according to the invention is any polypeptide comprising a naturally-occurring full-length ADP amino acid sequence or variant thereof that confers upon a vector expressing the ADP the ability to lyse a cell containing the vector such that replicated copies of the vector are released from the infected cell. A preferred full-length ADP comprises the ADP amino acid sequence encoded by Ad1, Ad2, Ad5 or Ad6. These naturally-occurring ADP sequences are set forth in SEQ ID NOS:5-8, respectively. ADP variants include fragments and deletion mutants of naturally-occurring adenovirus death proteins, as well as full-length molecules, fragments and deletion mutants containing conservative amino acid substitutions, provided that such variants retain the ability, when expressed by a vector inside a cell, to lyse the cell.

Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. Conservatively substituted amino acids can be grouped according to the chemical properties of their side chains. For example, one grouping of amino acids includes those amino acids having neutral and hydrophobic side chains (A, V, L, I, P, W, F, and M); another grouping is those amino acids having neutral and polar side chains (G, S, T, Y, C, N, and Q); another grouping is those amino acids having basic side chains (K, R, and H); another grouping is those amino acids having acidic side chains (D and E); another grouping is those amino acids having aliphatic side chains (G, A, V, L, and I); another

grouping is those amino acids having aliphatic-hydroxyl side chains (S and T); another grouping is those amino acids having amine-containing side chains (N, Q, K, R, and H); another grouping is those amino acids having aromatic side chains (F, Y, and W); and another grouping is those amino acids having sulfur-containing side chains (C and M). Preferred conservative amino acid substitutions groups are: R-K; E-D, Y-F, L-M; V-I, and Q-H.

As used herein, an ADP variant can also include modifications of a naturally-occurring ADP in which one or more amino acids have been inserted, deleted or replaced with a different amino acid or a modified or unusual amino acid, as well as modifications such as glycosylation or phosphorylation of one or more amino acids so long as the ADP variant containing the modified sequence retains cell lysing activity.

As described below, the inventors herein performed a structure-function analysis of ADP that defined specific domains in ADP required to promote cell death. Using this information, when combined with known recombinant DNA and cloning methodology, it is believed the skilled artisan can readily construct ADP variants of a naturally-occurring adenovirus death protein and test them for cell lysing activity. A preferred ADP deletion mutant comprises an ADP amino acid sequence from any of the deletion mutants *dI716*, *dI715*, *dI714* and *dI737*, whose ADP sequences are set forth in SEQ ID NOS:9-12, respectively).

Where the vector is derived from a virus, it is preferred that the virus lack expression of one or more viral proteins involved in avoiding host anti-viral defenses such as immune-mediated inflammation and/or apoptosis of infected cells. For example, adenovirus contains a cassette of genes that prevents killing of Ad-infected cells by the immune system (Wold et al., *Semin. Virol.*, 1998 (8:515-523, 1998). The E3-14.7K protein and the E3 RID (Receptor Internalization and Degradation) protein, which is a complex consisting of RID $\alpha$  and RID $\beta$ , inhibit apoptosis of Ad-infected cells induced by tumor necrosis factor (TNF) and the Fas ligand which are expressed on, or secreted by, activated macrophages, natural killer (NK) cells, and cytotoxic lymphocytes (CTLs) (Tollefson et al., *Nature* 392:727-730, 1998). The E3-gp19K protein inhibits CTL-killing of infected cells by blocking transport of MHC class I antigens to the cell surface (Wold et al., *supra*). Thus, it is believed that infection of tumor cells by such viral vectors will stimulate infiltration of inflammatory cells and lymphocytes into the tumor, and will not prevent infected tumor cells from apoptosis induced by cytolytic cells of the immune system, or against apoptosis inducing cytokines. For example, it is known that when mice are infected with Ad mutants lacking the E3 gp19K, RID and 14.7K proteins there is a dramatic increase (as compared to E3-positive Ad) in infiltration of inflammatory cells and lymphocytes into the infected tissue (Sparer et al., *J. Virol.* 70:2431-2439, 1996). A similar infiltration of tumors infected by an ADP-expressing viral vector of

the invention would be expected to further promote destruction of the tumor by adding an immune system attack to the ADP-mediated killing activity. For example, it is believed that the viral infection will stimulate formation of tumor-specific CTL's that can kill neoplastic cells not only in the tumor but also ones that have metastasized. In addition, it is also  
5 expected that vector-specific CTL's will be generated which could attack vector-infected cells if the vector spreads away from the tumor into normal cells. Because viral vectors overexpressing ADP will spread rapidly through the tumor, it is believed these immune mechanisms will have little effect on spread of the vector.

Where the vector is a recombinant adenovirus, it is preferred that the adenovirus lack  
10 expression of each of the E3 gp19K, RID, and 14.7K proteins. By "lack expression" and "lacking expression" of a protein(s), it is meant that the viral genome contains one or more mutations that inactivates expression of a functional protein, i.e., one having all the functions of the wild-type protein. The inactivating mutation includes but is not limited to substitution or deletion of one or more nucleotides in the encoding gene(s) that prevents expression of  
15 functional transcripts or that results in transcripts encoding nonfunctional translation products. A particularly preferred way to inactivate expression of the Ad E3 gp19K, RID, and 14.7K proteins is by deleting the E3 region containing the genes encoding these proteins. Preferably, one or both of the E3 genes encoding the E3 6.7K and 12.5K proteins are also deleted because, as discussed in the Examples below, it is believed that deletion of most or all  
20 of the E3 genes other than the ADP gene facilitates overexpression of ADP mRNA by reducing competition for splicing of the major late pre-mRNAs. Preferred Ad vectors containing an E3 deletion that overexpress ADP are GZ1 (SEQ ID NO:3) and GZ3 (SEQ ID NO:4), whose construction and properties are described in the Examples below.

The invention also provides ADP-expressing vectors whose replication is restricted to  
25 dividing cells. Any means known to provide such a replication-restricted phenotype may be used. For example, WO 96/40238 describes microbes that preferentially invade tumor cells as well as methods for identifying and isolating bacterial promoters that are selectively activated in tumors. It is also contemplated that expression of one or more vector proteins essential for replication can be placed under the control of the promoter for a cellular gene  
30 whose expression is known to be upregulated in neoplastic cells. Examples of such genes include but are not limited to: the breast cancer markers mammaglobin (Watson et al., *Oncogene* 16:817-824, 1998); BRCA1 (Norris et al., *J. Biol. Chem.* 270:22777-22782, 1995) *her2/neu* (Scott et al., *J. Biol. Chem.* 269:19848-19858, 1994); prostate specific antigen (U.S. Patent 5,698,443); surfactant protein B for lung alveoli (Yan et al., *J. Biol. Chem.* 270:24852-  
35 24857, 1995); factor VII for liver (Greenberg et al., *Proc. Natl. Acad. Sci. USA* 92:12347-12351, 1995); and survivin for cancer in general (Li et al., *Nature* 396:580-584). Where the

vector is an adenovirus, it is contemplated that such tumor-specific promoters can be substituted for the E4 promoter. Because E4 gene products are essential for Ad replication, placing their expression under the control of a tumor-specific promoter should restrict replication of the vector to tumor cells in which the promoter is activated.

5 Another strategy for restricting replication of ADP-expressing Ad vectors to neoplastic cells is exemplified by the KD1 (SEQ ID NO:1), KD2 (SEQ ID NO:13) and KD3 (SEQ ID NO:2) vectors, whose construction and properties are described in the Examples below. This strategy exploits a pre-existing Ad5 mutant in the E1A gene, named *d1101/1107* (Howe et al., *Proc. Natl. Acad. Sci.*, 87:5883-5887, 1990), also referred to herein as *d101/07*,  
10 and which can only grow well in cancer cells. The role of E1A is to drive cells from the G<sub>0</sub> and G<sub>1</sub> phases of the cell cycle into S-phase. This is achieved by two mechanisms, one involving pRB (and family members), and the other involving p300 and the related protein CBP (DePinho, R.A., *Nature* 391:533-536, 1998). One domain in E1A binds members of the pRB family. pRB normally exists in the cell as a complex with the transcription factor E2F-1  
15 and E2F family members (E2F), tethered via E2F to E2F binding sites in promoters of cells expressed in S-phase. Here, pRB acts as a transcriptional co-repressor. E1A binding to pRB relieves this repression, and causes the release of E2F from pRB/E2F complexes. Free E2F then activates promoters of genes expressed in S-phase, e.g. thymidine kinase, ribonucleotide reductase, etc. Another domain in E1A binds the p300/CBP transcription adaptor protein  
20 complex. p300/CBP is a transcriptional co-activator that binds many different transcription factors and accordingly is targeted to promoters. p300/CBP has intrinsic histone acetyltransferase activity. E1A binding to p300/CBP is believed to inhibit this histone acetyltransferase activity, allowing acetylation of histones and repression of transcription (Chakravarti et al., *Cell* 96:393-403, 1999; Hamamori et al., *Cell* 96:405-413, 1999).  
25 Conceivably, some of the genes that are repressed as a result of E1A interacting with p300/CBP to play a role in blocking the cell cycle, although this is not known. Cancer cells are cycling, so they have free E2F and presumably some p300/CBP-regulated genes are repressed. Consistent with these ideas, E1A must bind both p300/CBP and the pRB family in order to transform primary cells to a constitutively cycling state (Howe et al., *supra*). The  
30 mutant *d101/07* lacks both the p300/CBP- and pRB-binding domains and, as expected, it replicates very poorly in non-dividing "normal" cells or serum-starved cancer cells, but well in growing cancer cells. As described below, the growth of the KD1 and KD3 vectors, which contain the *d101/07* E1A mutation, is very much better in dividing cancer cells as compared to non-dividing cells. Because the *d101/07* mutant is completely defective in oncogenic  
35 transformation of rat cells (Howe et al., *supra*), vectors according to the invention that contain



this E1A mutation cannot induce cancer in humans (remote as that may be) through an E1A-dependent mechanism.

The invention also includes vectors overexpressing ADP whose replication is restricted to specific tissues by placing expression of one or more proteins essential for replication under the control of a tissue specific promoter and/or a tumor specific promoter. A number of tissue-specific and/or tumor specific promoters have been described in the art. Non-limiting examples include the surfactant protein B promoter, which is only active in cells containing the TTF1 transcription factor (i.e., type II alveolar cells (Yan et al., *supra*)), as described in U.S. Patent 5,466,596 to Breitman et al., which directs gene expression specifically in cells of endothelial lineage; prostate specific antigen which is expressed in prostate cells (Rodriguez et al., *supra*); human telomerase protein (hTERT) promoter (see, e.g., U.S. Patent No. 6,054,575); and human alpha-lactalbumin gene which is expressed in breast cancer cells (Anderson et al., *Gene Therapy* 6:854-864, 1999). Many other tissue-specific, tumor specific, or tissue-preferred enhancer/promoters have been reported (Miller and Whelan, *Human Gene Therapy* 8:803-815, 1997). As exemplified with the surfactant protein B promoter in Examples 6 and 10, vectors expressing tissue-specific promoters would be expected to show tissue specificity in viral replication, viral spreading, cell lysis, and tumor suppression.

Replication of vectors according to the invention can also be controlled by placing one or more genes essential for vector replication under the control of a promoter that is activated by an exogenous inducing agent, such as metals, hormones, antibiotics, and temperature changes. Examples of such inducible promoters include but are not limited to metallothionein promoters, the glucocorticoid promoter, the tetracycline response promoter, and heat shock protein (hsp) promoters such as the hsp 65 and 70 promoters.

The invention also provides compositions comprising a recombinant vector that overexpresses ADP in an amount effective for promoting death of neoplastic cells and a method comprising administering a therapeutically effective amount of the vector to a neoplastic cell in a patient. It is believed the compositions and methods of the present invention are useful for killing neoplastic cells of any origin and include neoplastic cells comprising tumors as well as metastatic neoplastic cells.

It is also contemplated that ADP-expressing viral vectors can be administered to neoplastic cells along with a replication-defective virus that expresses an anti-cancer gene product. For example, many replication-defective E1<sup>-</sup> Ad vectors for use in cancer therapy are well characterized. A limitation of replication-defective vectors is that they only synthesize the therapeutic protein in the cell they initially infect, they cannot spread to other cells. Also, since the genome does not replicate, transcription can only occur from the input

genomes, and this could be as low as one copy per cell. In contrast, the genome of replication-competent Ad vectors are amplified by about  $10^4$  in the cell that was initially infected, providing more templates for transcription. More amplification is achieved as the vector spreads to other cells. By combining replication-defective viral vectors expressing an anti-cancer gene product with replication-competent viral vectors described herein, it is expected that the result will be template amplification and rapid spread of both vectors to surrounding cells. For example, with Ad-based vectors, the burst size for each vector should be large,  $\sim 10^4$  PFU/cell, so the probability of co-infection of surrounding cells by both vectors will be high. Thus, both the replication-competent and replication-defective vectors should spread simultaneously through the tumor, providing even more effective anti-cancer therapy.

As an alternative method of delivering an anti-cancer gene product with an ADP overexpressing Ad vector, the anti-cancer gene can be engineered into any of the ADP overexpressing replication-competent vectors described herein, in order to provide both the ADP and the anti-cancer function in a single vector. The anti-cancer gene can be engineered into any appropriate location of the vector, as can be easily determined by the skilled artisan. For example, the anti-cancer gene can be engineered into the E3 region.

Expression of the anti-cancer gene product encoded by the replication-defective vector can be under the control of either constitutive, inducible or cell-type specific promoters. The anti-cancer gene product can be any substance that promotes death of a neoplastic cell. The term "gene product" as used herein refers to any biological product or products produced as a result of the biochemical reactions that occur under the control of a gene. The gene product can be, for example, an RNA molecule, a peptide, a protein, or a product produced under the control of an enzyme or other molecule that is the initial product of the gene, i.e., a metabolic product. For example, a gene can first control the synthesis of an RNA molecule which is translated by the action of ribosomes into a prodrug converting enzyme which converts a nontoxic prodrug administered to a cancer patient to a cell-killing agent; the RNA molecule, enzyme, and the cell-killing agent generated by the enzyme are all gene products as the term is used here. Examples of anti-cancer gene products include but are not limited to cell-killing agents such as apoptosis-promoting agents and toxins; prodrug converting enzymes; angiogenesis inhibitors; and immunoregulatory molecules and antigens capable of stimulating an immune response, humoral and/or cellular, against the neoplastic cell.

Apoptosis-promoting agents include but are not limited to the pro-apoptotic members of the BCL-2 family such as BAX, BAD, BID and BIK, as well as antisense molecules which block expression of anti-apoptotic members of the BCL-2 family. Examples of immunoregulatory molecules are cytokines such as tumor necrosis factor, Fas/Apo1/CD95

ligand; tumor necrosis factor related apoptosis inducing ligand, interleukins, macrophage activating factor and interferon  $\gamma$ . Angiogenesis inhibitors include but are not limited to endostatin and angiostatin. Toxins include but are not limited to tumor necrosis factor, lymphotoxin, the plant toxin ricin, which is not toxic to humans due to the lack of ricin  
5 receptors in animal cells, and the toxic subunit of bacterial toxins. Examples of pro-drug converting enzymes and pro-drug combinations are described in WO 96/40238 and include thymidine kinase and acyclovir or gancyclovir; and bacterial cytosine deaminase and 5-fluorocytosine.

The therapeutic or pharmaceutical compositions of the present invention can be  
10 administered by any suitable route known in the art including for example by direct injection into a tumor or by other injection routes such as intravenous, subcutaneous, intramuscular, transdermal, intrathecal and intracerebral. Administration can be either rapid as by injection or over a period of time as by slow infusion or administration of slow release formulation. For treating tissues in the central nervous system, administration can be by injection or  
15 infusion into the cerebrospinal fluid (CSF). When it is intended that a recombinant vector of the invention be administered to cells in the central nervous system, administration can be with one or more agents capable of promoting penetration of the vector across the blood-brain barrier. Preferably, vectors of the invention are administered with a carrier such as liposomes or polymers containing a targeting moiety to limit delivery of the vector to targeted cells.  
20 Examples of targeting moieties include but are not limited to antibodies, ligands or receptors to specific cell surface molecules.

Compositions according to the invention can be employed in the form of pharmaceutical preparations. Such preparations are made in a manner well known in the pharmaceutical art. One preferred preparation utilizes a vehicle of physiological saline  
25 solution, but it is contemplated that other pharmaceutically acceptable carriers such as physiological concentrations of other non-toxic salts, five percent aqueous glucose solution, sterile water or the like may also be used. It may also be desirable that a suitable buffer be present in the composition. Such solutions can, if desired, be lyophilized and stored in a sterile ampoule ready for reconstitution by the addition of sterile water for ready injection.  
30 The primary solvent can be aqueous or alternatively non-aqueous.

The carrier can also contain other pharmaceutically-acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation. Similarly, the carrier may contain still other pharmaceutically-acceptable excipients for modifying or maintaining release or absorption or  
35 penetration across the blood-brain barrier. Such excipients are those substances usually and customarily employed to formulate dosages for parenteral administration in either unit dosage

or multi-dose form or for direct infusion into the cerebrospinal fluid by continuous or periodic infusion.

It is also contemplated that certain formulations containing ADP-expressing vectors are to be administered orally. Such formulations are preferably encapsulated and formulated with suitable carriers in solid dosage forms. Some examples of suitable carriers, excipients, and diluents include lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, alginates, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, gelatin, syrup, methyl cellulose, methyl- and propylhydroxybenzoates, talc, magnesium, stearate, water, mineral oil, and the like. The formulations can additionally include lubricating agents, wetting agents, emulsifying and suspending agents, preserving agents, sweetening agents or flavoring agents. The compositions may be formulated so as to provide rapid, sustained, or delayed release of the active ingredients after administration to the patient by employing procedures well known in the art. The formulations can also contain substances that diminish proteolytic degradation and promote absorption such as, for example, surface active agents.

The specific dose is calculated according to the approximate body weight or body surface area of the patient or the volume of body space to be occupied. The dose will also be calculated dependent upon the particular route of administration selected. Further refinement of the calculations necessary to determine the appropriate dosage for treatment is routinely made by those of ordinary skill in the art. Such calculations can be made without undue experimentation by one skilled in the art. Exact dosages are determined in conjunction with standard dose-response studies. It will be understood that the amount of the composition actually administered will be determined by a practitioner, in the light of the relevant circumstances including the condition or conditions to be treated, the choice of composition to be administered, the age, weight, and response of the individual patient, the severity of the patient's symptoms, and the chosen route of administration. Dose administration can be repeated depending upon the pharmacokinetic parameters of the dosage formulation and the route of administration used.

The invention also contemplates passively immunizing patients who have been treated with a viral vector overexpressing ADP. Passive immunization can include administering to the patient antiserum raised against the viral vector, or gamma-globulin or vector-specific purified polyclonal or monoclonal antibodies isolated from the antiserum. Preferably, the patient is passively immunized after a time period sufficient for the viral vector to replicate in and spread through the tumor.

Preferred embodiments of the invention are described in the following examples. Other embodiments within the scope of the claims herein will be apparent to one skilled in the

art from consideration of the specification or practice of the invention as disclosed herein. It is intended that the specification, together with the examples, be considered exemplary only, with the scope and spirit of the invention being indicated by the claims which follow the examples.

5

#### Example 1

This example illustrates the construction and characterization of the KD1 and KD3 anti-cancer vectors.

To construct KD1, the inventors deleted the entire E3 region of a unique plasmid, leaving behind only a unique *PacI* site for cloning. The starting plasmid was pCRII, purchased from Invitrogen, containing the Ad5 BamHIA fragment having a deletion of all the E3 genes; the E3 deletion is identical to that for KD1 and GZ3, the sequences of which are given in SEQ ID NO:1 and SEQ ID NO:4, respectively. The ADP gene from Ad5 was cloned into the *PacI* site, then built into the E3 region of the genome of the Ad5 E1A mutant named *dI01/07*. This was done by co-transfecting into human embryonic kidney 293 cells the aforementioned BamHIA fragment containing the ADP gene together with the overlapping EcoRIA restriction fragment obtained from *dI01/07*. Complete viral genomes are formed within the cell by overlap recombination between the Ad sequences in the BamHIA fragment in the plasmid and the EcoRIA fragment. KD3 was constructed in the same way except the E3 gene for the 12.5K protein was retained in the starting plasmid. A vector named KD2, which marginally overexpress ADP, was also prepared. Plaques of each recombinant Ad were picked, screened, purified, expanded into CsCl-banded stocks, sequenced, titered, and characterized. GZ1 and GZ3 are Ad vectors that are identical to KD1 and KD3, respectively, except that GZ1 and GZ3 have wild-type E1A sequences as found in AD5 or in the Ad5 mutant *dI309*. GZ1 and GZ3 were constructed as described for KD1 and KD3 except that the EcoRIA fragment of Ad5 was used for GZ1 and GZ3.

KD1 and KD3 were characterized in cell culture by infecting the human A549 lung carcinoma cell line with high titer ( $1-8 \times 10^{10}$  plaque forming units [PFU] per ml) virus stocks of one of these recombinant vectors, or with one of the control viruses *dI01/07*, *dI309*, *dI327*, and Ad5 (wt). Fifty PFU per cell were used for each virus. The descriptions of these viruses as well as some other viruses used in these examples are presented in Table 1.

Table 1: Description of mutations in viruses:

us	RNA				REGION	
	E1	VA	E3	E4		
101/1107	<i>d</i> /1101: deletion of Ad5 bp 569-634 <i>d</i> /1107: deletion of Ad5 bp 890-928	From <i>d</i> /309 deletion of Ad5 bp 10594-10595	From <i>d</i> /309 deletion of Ad5 bp 28597-28602; deletion-substitution Ad5 bp 3005-30750, insert 642 bp DNA of unknown origin	wild type		
11	<i>d</i> /1101: deletion of Ad5 bp 569-634 <i>d</i> /1107: deletion of Ad5 bp 890-928	From <i>d</i> /309 deletion of Ad5 bp 10594-10595	deletion of Ad5 bp 27858-2760, TAA inserted; deletion of Ad5 bp 27982-28134; deletion of Ad5 bp 28395-29397, insert CCTTAATTAAA; deletion of Ad5 bp 29783-30883, insert TTAATTAAAGG	wild type		
12	<i>d</i> /1101: deletion of Ad5 bp 569-634 <i>d</i> /1107: deletion of Ad5 bp 890-928	From <i>d</i> /309 deletion of Ad5 bp 10594-10595	<i>d</i> /309 background, gp19K mutated deletion of Ad5 bp 28597-28602; deletion-substitution Ad5 bp 3005-30750, insert 642 bp DNA of unknown origin; deletion of Ad5 bp 28788-28789, insert TTAATTAA	wild type		
13	<i>d</i> /1101: deletion of Ad5 bp 569-634 <i>d</i> /1107: deletion of Ad5 bp 890-928	From <i>d</i> /309 deletion of Ad5 bp 10594-10595	deletion of Ad5 bp 28598-29397; deletion of Ad5 bp 29783-30469	wild type		
14	wt	wild type	deletion of Ad5 bp 27858-2760, TAA inserted; deletion of Ad5 bp 27982-28134; deletion of Ad5 bp 28395-29397, insert CCTTAATTAAA; deletion of Ad5 bp 29783-30883, insert TTAATTAAAGG	wild type		

	wild type	wild type	deletion of Ad5 bp 28598-29397; deletion of Ad5 bp 29783-30469	wild type
01/1107-	<i>d11101</i> : deletion of Ad5 bp 569-634 <i>d11107</i> : deletion of Ad5 bp 890-928	From <i>d1309</i> deletion of Ad5 bp 10594-10595	From <i>d1309</i> deletion of Ad5 bp 28597-28602; deletion-substitution Ad5 bp 3005-30750, insert 642 bp DNA of unknown origin	E4 promoter deletion-substitution: deletion of Ad5 bp 35623-35775, insert SP-B 500 promoter flanked by BstI 107I sites
1-SPB	<i>d11101</i> : deletion of Ad5 bp 569-634 <i>d11107</i> : deletion of Ad5 bp 890-928	From <i>d1309</i> deletion of Ad5 bp 10594-10595	deletion of Ad5 bp 27848-2760, TAA inserted; deletion of Ad5 bp 27982-28134; deletion of Ad5 bp 28395-29397, insert CCTTAATTAAA; deletion of Ad5 bp 29783-30883, insert TTAAATTAAAGG	E4 promoter deletion-substitution: deletion of Ad5 bp 35623-35775, insert SP-B 500 promoter flanked by BstI 107I sites
1-SPB	<i>d11101</i> : deletion of Ad5 bp 569-634 <i>d11107</i> : deletion of Ad5 bp 890-928	From <i>d1309</i> deletion of Ad5 bp 10594-10595	deletion of Ad5 bp 28598-29397; deletion of Ad5 bp 29783-30469	E4 promoter deletion-substitution: deletion of Ad5 bp 35623-35775, insert SP-B 500 promoter flanked by BstI 107I sites

Using a polymerase chain reaction (PCR)-based protocol, an in-frame stop codon was introduced into the gene for the E3-gp19K protein in the E3 region of the Ad5 mutant *dI309* (Jones and Shenk, *Cell* 17:683-689, 1979). The mutagenesis was conducted using a *SunI*-*Bst*1107I fragment, nucleotides 28,390 to 29,012 in the Ad5 genome, which was then substituted for the equivalent fragment in *dI309*. *dI01/07* is the parent for KD1 and KD3. In turn, the Ad5 mutant named *dI309* is the parent of *dI01/07*, i.e. *dI309* is identical to *dI01/07* except that *dI309* does not have the E1A mutation. Both *dI01/07* and *dI309* have deletions of the genes for the E3 RID $\alpha$ , RID $\beta$  and 14.7K proteins but retain the gene for ADP. The Ad5 mutant *dI327* has wild-type E1A, it lacks the gene for ADP, and it lacks all other E3 genes except the one for the 12.5K protein.

At 24 and 36 hours post-infection (h p.i.), proteins were extracted from the A549 cells and analyzed for ADP by immunoblot using a rabbit antiserum against ADP (Tollefson et al., *J. Virol.* 66:3633-3642, 1992). The results are shown in Figure 2. Much more ADP was detected at 24 and 36 h p.i. in KD1- and KD3-infected cells than in cells infected with *dI01/07*. Also, much more ADP was synthesized by GZ1 and GZ3 than *dI309* or the other viruses. Most importantly, KD1, KD3, GZ1, and GZ3 expressed much more ADP at 24 h p.i. than did *dI01/07* or *dI309* (Fig. 2). This result is consistent with an observation discussed below that the cells infected with KD1, KD3, GZ1, or GZ3 lyse faster, and that these viruses spread from cell to cell faster than *dI01/07* or *dI309*. It is noteworthy that KD1, KD3, GZ1, and GZ3 express much more ADP at 24 and 36 h p.i. than the Ad5 mutant *dI1520* (Fig. 2); *dI1520* is the original name given to ONYX-015 (Heise et al., *Nature Medicine* 3:639-645, 1997). As expected, no ADP was detected in cells infected with *pm734.1* (Fig. 2), a mutant that lacks amino acids 1 to 48 in ADP (Tollefson et al., *J. Virol.* 70:2296-2306, 1996). Expression of the E1A proteins by *dI01/07*, KD1, KD2, and KD3 was slightly less than by Ad5, *dI309*, or *dI327*, and as expected from the *dI01/07* deletion, the proteins were smaller (Fig. 3A). *dI327* is isogenic with *dI324* (Thimmappaya et al., 1982 *Cell* 31:543-51, 1983), and it lacks the gene for ADP and all other E3 proteins except the 12.5K protein.

The amount of ADP detected in the KD1 and KD3 infected cells is significantly higher than the amount detected in the *dI309* infected cells (Fig. 2). If one takes into consideration the fact that the viruses with the E1A mutation replicate somewhat slower, as evidenced in by the delayed appearance of the late proteins (Fig. 3B), it is clear that KD1 and KD3 express much more ADP per viral genome present in the cell than *dI309*. This finding is supported by the fact that when A549 cells are coinfecting with a virus containing the E1A mutation and *dI327*, which lacks ADP but has wild-type E1A, the replication rates of the E1A mutant viruses speed up, as indicated by earlier appearance of late proteins (compare Figs. 3B



and 3D). Thus, *dI327* complements the E1A mutation. In conclusion, these experiments demonstrate that ADP is dramatically overexpressed by KD1, KD3, GZ1, and GZ3. ADP is marginally overexpressed by KD2 (not shown).

#### Example 2

5        This example illustrates that KD1 and KD3 lyse cells more rapidly and spread from cell-to cell faster than other adenoviruses.

      The ability of KD1 and KD3 to lyse cells was examined by a trypan blue exclusion cell viability assay which was performed essentially as described by Tollefson et al., *J. Virol.* 70:2296-2306, 1996. In brief, A549 cells were mock-infected or infected with 20 PFU/cell of  
10    KD1, KD3, *dI01/07*, *dI327* or *dI309*. At various days p.i., the number of viable cells was determined using a hemocytometer (600 to 1000 cells were counted per time point) and the results are shown in Fig. 4.

      Only 25% of the KD1-infected cells and 9% of the KD3-infected cells were alive at 5 days p.i. as compared to 44% of cells infected with *dI01/07*, which has the same E1A  
15    mutation as KD1 and KD3. The KD1 and KD3 vectors also lysed cells faster than *dI309*, which has a wild-type E1A region. When infected with *dI327* (ADP<sup>+</sup>, E1A<sup>+</sup>), 94% of the cells were alive after 5 days. When cell lysis was estimated by release of lactate dehydrogenase, KD1 and KD3 once again lysed cells faster than *dI01/07* and *dI309*, and *dI327* caused little cell lysis (data not shown). Thus, ADP is required for efficient cell lysis, and over-expression  
20    of ADP increases the rate of cell lysis.

      As another means to measure cell lysis and to examine virus replication in cancer cells, separate groups of A549 cells were infected with 20 PFU/cell of KD1, KD3, *dI01/07*, or *dI309* and the amount of intracellular and extracellular virus was determined by plaque assay on A549 cells. At 2 days p.i., the total amount of virus formed in each group was similar, 2-4  
25    x 10<sup>8</sup> PFU/ml, indicating that replication of all the viruses is similar. However, when the ratio of extracellular to intracellular virus was calculated, the value for KD1 and KD3 was 2-3 logs higher than for Ad5, *dI309*, or *dI01/07* (data not shown). Thus, virus is released much more rapidly from cells infected with KD1 and KD3, which overexpress ADP, than with viruses expressing wild-type amounts of ADP.

30        The ability of KD1 and KD3 to spread from cell-to-cell was measured in a "cell spreading" assay. In this assay monolayers of A549 cells in a 48 well culture dish were mock-infected or infected with 10<sup>-3</sup>, 10<sup>-2</sup>, 10<sup>-1</sup>, 10<sup>0</sup>, or 10 PFU/cell of *dI327*, *dI309*, Ad5, *dI01/07*, KD1 or KD3. At low PFU/cell, the viruses must go through two or three rounds of replication in order to infect every cell in the monolayer. At 1.0 and 10 PFU/cell, the  
35    monolayer should be destroyed by the virus that initially infected the cells. To assess the

amount of spread in the monolayers by 7 days p.i., crystal violet, which stains live cells but not dead cells, was added to the monolayers. The results are shown in Fig. 5.

Remarkably, at 7 days p.i., the monolayer was virtually eliminated by KD1 and KD3 at  $10^{-3}$  PFU/cell, whereas 1.0 PFU/cell was required with *dI01/07*, *dI309* and Ad5. This result attests to the potency of ADP in mediating cell lysis and virus spread in A549 cells. KD1 and KD3 are also more effective than *dI01/07* in killing other types of human cancer cell lines (most purchased from the American Type Culture Collection [ATCC]) as determined in this cell spreading assay. KD1 and/or KD3 killed HeLa (cervical carcinoma), DU145 (prostate), and pC3 (prostate) cells at  $10^{-2}$  PFU/cell, ME-180 (cervix) and Hep3B (liver) at  $10^{-1}$  PFU/cell, and U118 (glioblastoma) and U373 (glioblastoma) at 10 PFU/cell. From 10- to 100-fold more *dI01/07* was required to kill these cells (data not shown). These results indicate that KD1 and KD3 may be effective against many types of cancer.

An important aspect of the finding that ADP overexpressing vectors lyse cells at very low multiplicities of infection is that the multiplicity of infection in human tumors is likely to be low at sites distal to the sight of vector injection or distal to blood vessels that carry the vector to the tumor. Thus, ADP overexpressing vectors have an advantage over vectors that express less ADP or no ADP at all.

### Example 3

This example illustrates that KD1 and KD3 replicate poorly in non-growing non-cancerous cells. The replication phenotype of KD1 and KD3 was evaluated using "normal" HEL-299 human fibroblast cells, either growing in 10% serum or rendered quiescent using 0.1% serum. All Ads should replicate well in growing cells, but viruses with the *dI01/07* E1A mutation should do poorly in quiescent cells because E1A is required to drive them out of  $G_0$ . *dI309*, which has wild-type E1A, should replicate well in both growing and growth-arrested cells.

Cells were infected with 100 PFU/cell of KD1, KD3, *dI01/07*, or *dI309*. At different days p.i., virus was extracted and titered. In 10% serum, KD1, KD3, and *dI01/07* replicated well, reaching titers of  $10^6$ - $10^7$  PFU/ml, only slightly less than *dI309* (Fig. 6). However, in quiescent cells, replication of KD1, KD3, and *dI01/07* was 1.5-2 logs lower than in growing cells, ranging from  $10^4$  to  $2 \times 10^5$  PFU/ml. The titer of *dI309* reached  $10^7$  PFU/ml, nearly the level achieved in growing cells. At 10 days p.i., quiescent HEL-299 cell monolayers infected with 100 PFU/cell of KD1, KD3, or *dI01/07* were intact, whereas those infected with *dI309* or *dI327*, which have wild-type E1A, showed strong typical Ad cytopathic effect indicative of cell death (data not shown). Thus, replication of KD1 and KD3 is severely restricted to growing cell lines.

The restriction associated with the *dl01/07* E1A mutation was also tested in primary human cells (purchased from Clonetics) growing as monolayers. Bronchial epithelial cells (Fig. 7) and small airway epithelial cells were not killed by 10 PFU/cell of KD1, KD3, or *dl01/07* at 5 days p.i., whereas they were killed by 10 PFU/cell of *dl309* or *dl327* (data not shown). Lung endothelial cells also were not killed after 10 days by KD1, KD3, or *dl01/07* at 10 PFU/cell, but they were killed by 1 PFU/cell of *dl309*. These monolayers were subconfluent when initially infected, then grew to confluency. The exciting result here is that although these primary cells were growing, they did not support replication in this time frame and were not killed by KD1 or KD3. Thus, it is believed these vectors will be restricted to cancerous cells, and will have little to no effect on cells such as basal cells that are normally dividing in the body. In addition, it is unlikely that KD1 and KD3 will affect dividing leukocytes because such cells are poorly infected by Ad.

In summary, the above experiments demonstrate that KD1 and KD3 lyse cancer cells, spread from cell-to-cell rapidly, and replicate poorly in quiescent and non-cancerous cells. These properties should make them useful in anti-cancer therapy.

#### Example 4

This example illustrates that KD1 and KD3 inhibit the growth of human tumors in an animal model.

We could not evaluate mouse or rat tumors in normal mice or rats because they are totally non-permissive. Human cancer cell lines growing in nude mice have been used by Onyx Pharmaceuticals (Richmond, CA) to evaluate the efficacy of ONYX-015, an Ad vector lacking expression of the E1B 55 kDa protein (Heise et al., *Nature Med.* 3:639-645, 1997). We have found that A549 cells, which were used in many of our cell culture studies, form excellent rapidly growing solid tumors when injected subcutaneously into nude mice. The average tumor reaches ca. 500  $\mu$ l in four weeks, and is encapsulated, vascularized, and attached to the mouse skin (usually) or muscle.

Nude mice were inoculated into each hind flank with  $2 \times 10^7$  A549 cells. After 1 week tumors had formed, ranging in size from about 20  $\mu$ l to 50  $\mu$ l. Individual tumors were injected three days later, and at subsequent weeks for 4 weeks (total of 5 injections), with 50  $\mu$ l of buffer or 50  $\mu$ l of buffer containing  $5 \times 10^7$  PFU of *dl309*, *dl01/07*, KD1, KD3, or *pm734.1*, with a total virus dose per tumor of  $3 \times 10^8$  PFU. The mutant *pm734.1* lacks ADP activity due to two nonsense mutations in the gene for ADP, but all other Ad proteins are expected to be synthesized at wild-type levels (Tollefson et al., *J. Virol.* 70:2296-2306, 1996). The efficacy of each virus (or buffer) was tested on six tumors. At weekly intervals, the length (L) and width (W) of tumors were measured using a Mitutoyo digital caliper. Tumor

volumes were calculated by multiplying  $L \times W \times W/2$ . This value was divided by the tumor volume at the time of the initial virus injection, the fold-increase in tumor growth was calculated, and the average for the six tumors was graphed.

As shown in Fig. 8A, tumors that received buffer continued to grow, increasing about 14-fold by 5 weeks. In contrast, tumors injected with *d/309*, which expresses normal amounts of ADP and lacks the E3 RID and 14.7K and proteins, only grew about 2.5-fold by 5 weeks. With *pm734.1*, which lacks ADP, the tumors grew as well as those that received buffer. Thus, *d/309* markedly decreases the rate of tumor growth, and ADP is required for this decrease. Tumors inoculated with *d/01/07* grew about 8-fold over 5 weeks. Since *d/01/07* is identical to *d/309* except for the E1A mutation, this result indicates that the E1A mutation significantly reduces the ability of Ad to prevent growth of the tumors. This effect is probably due to a reduction in virus replication in the tumors resulting in lower ADP expression, but it could also reflect other properties of E1A in the tumor cells, e.g. the inability of the mutant E1A proteins to induce apoptosis. Most importantly, tumors inoculated with KD1 or KD3 only grew about 2.5-fold. Thus, the overexpression of ADP by KD1 and KD3 allows KD1 and KD3 to reduce tumor growth to a rate markedly slower than *d/01/07* (their parental control virus), and even to a rate similar to that of *d/309*.

The finding that KD1 and KD3 are as effective as wild-type Ad (i.e. *d/309*) in reducing the rate of A549 tumor growth is highly significant in the context of cancer treatment, inasmuch as KD1 and KD3 are restricted to cancer cells whereas wild-type Ad does not have such a restriction.

The tumors in Fig. 8A received five injections of vectors, but only one dose of vector, in this case  $5 \times 10^8$  of each of KD3 or GZ3, is sufficient to significantly reduce the rate of A549 tumor growth (Fig. 8B).

We have also found that KD1 and KD3 reduce the rate of growth in nude mice of a human liver cancer cell line, Hep3B cells. These cells form rapidly growing tumors that are highly vascularized. Nude mice were inoculated into each hind flank with  $1 \times 10^7$  of Hep3B cells. After tumors reached about 100  $\mu$ l, they were injected twice per week for 3 weeks with 50  $\mu$ l of buffer or  $5 \times 10^7$  PFU of KD1, KD3, or *d/309*. There were typically 8-10 tumors per test virus. The tumor sizes were measured and the fold increase in size at 0 to 3.5 following the initial virus injection was graphed as described above for the A549 tumors. Tumors that received buffer alone grew 9-fold over 3 weeks and were projected to grow about 12-fold over 3.5 weeks (after 3 weeks the mice had to be sacrificed because the tumors were becoming too large) (Fig. 9). Tumors that received KD1 or KD3 grew about 4-fold, establishing that KD1 and KD3 reduce the growth of Hep3B tumors in nude mice. Tumors

that were injected with *dl*309 grew 2-fold (Fig. 9). The finding that KD1 and KD3 were somewhat less effective than *dl*309 is probably due to the fact that they do not grow as well as *dl*309 in Hep3B cells, as indicated by a cell spread assay in culture (data not shown). In any case, the important points are that KD1 and KD3 are effective against the Hep3B tumors, and that they contain the E1A mutation that limits their replication to cancer cells.

These results point to the potency of ADP as an anti-tumor agent when expressed in an Ad vector. It is highly probable that KD1 and KD3 will provide significant clinical benefit when used to infect tumors growing in humans.

#### Example 5

This example illustrates the use of replication-defective Ad vectors in combination with KD1 or KD3.

It is well established that replication-competent (RC) viruses complement replication-defective (RD) mutants. That is, when the same cell is infected, the competent virus will supply the protein(s) that cannot be made from the mutant genome, and both viruses will grow. To test the ability of KD1 and KD3 to complement RD viruses, two RD vectors expressing  $\beta$ -galactosidase were constructed. The first, named Ad- $\beta$ -gal, has a cDNA encoding  $\beta$ -gal under the control of the Rous Sarcoma Virus promoter substituted for the deleted E1 region. Ad- $\beta$ -gal also has the E3 region deleted, including the gene for ADP. The second, named Ad- $\beta$ -gal/FasL is identical to Ad- $\beta$ -gal, except that it also expresses murine FasL from the human cytomegalovirus promoter/enhancer. These vectors were constructed by overlap recombination in human 293 cells that constitutively express the Ad E1A and E1B genes and complement replication of the E1-minus vectors.

These RD vectors should infect and express  $\beta$ -gal in A549 cells, but should not replicate because the E1A proteins are lacking. However, the vectors should replicate when cells are co-infected with RC Ads. To prove this, A549 cells were infected with 10 PFU/cell of Ad- $\beta$ -gal alone, or with 10 PFU/cell of Ad- $\beta$ -gal plus 10 PFU/cell of KD1, KD3, *dl*01/07, *dl*309, or *dl*327. At 2 days p.i., virus was extracted and Ad- $\beta$ -gal titers determined by  $\beta$ -gal expression in A549 cells. The yields are shown in Table 2 below.

Table 2

Virus	Yield (blue plaques per ml)
Ad- $\beta$ -gal	$1 \times 10^2$
Ad- $\beta$ -gal + KD1	$2 \times 10^5$
Ad- $\beta$ -gal + KD3	$3 \times 10^5$
Ad- $\beta$ -gal + <i>d</i> /01/07	$4 \times 10^4$
Ad- $\beta$ -gal + <i>d</i> /309	$3 \times 10^5$
Ad- $\beta$ -gal + <i>d</i> /327	$3.0 \times 10^5$

The data in Table 2 indicate that the complementing viruses increased the yield of Ad- $\beta$ -gal by about  $10^3$ .

5           A key feature of KD1 and KD3 is that they spread from cell-to-cell faster than other Ads. Accordingly, they should complement the spread of Ad- $\beta$ -gal. To test this, an infectious center assay was conducted. A549 cells were infected with Ad- $\beta$ -gal plus KD1, KD3, or *d*/01/07. After 2 h, cells were collected, diluted, and seeded onto monolayers of fresh A549 cells. After 4 days, the cells were stained with X-gal and the results are shown in  
10 Fig. 10.

With Ad- $\beta$ -gal alone, only the originally infected cell (before seeding) should be stained, and the vector should not spread to other cells on the seeded monolayer. This was indeed the case. In monolayers seeded with A549 cells infected with Ad- $\beta$ -gal alone (dish shown in the top left of Fig. 10A) contained a number of individual blue cells (not visible in the print); examples are shown in the enlarged view Fig. 10B. However, when the  
15 monolayers were seeded with A549 cells coinfecting with Ad- $\beta$ -gal and KD1 or KD3, there were numerous "comets" of blue cells (Fig. 10A). Each comet represents Ad- $\beta$ -gal which has spread from one initially-infected cell. Most of the cells within a comet were stained with X-gal (Fig. 10C). Comets were also observed with *d*/01/07, but not to the extent of KD1 and  
20 KD3 (Fig. 10A). With *d*/327 (ADP), there was little spread from the originally infected cell (data not shown). In summary, KD1 and KD3 not only complement the replication of Ad- $\beta$ -gal, they also enhance its rapid spread.

It is expected that KD1 and KD3 will also complement and enhance the spread of RD vectors expressing anti-cancer therapeutic gene products, and this expectation can be readily

verified using the Ad- $\beta$ -gal/FasL in replication and infectious center assays as described above.

KD1 and KD3 not only complement the replication of RD vectors in cell culture, they also do so in Hep3B tumors growing in the hind flanks of nude mice. The RD vector used was AdLuc, an Ad that lacks the E1 and E3 regions, and has inserted into the E1 region an expression cassette where the firefly luciferase gene is expressed from the Rous Sarcoma Virus promoter (Harrod et al., *Human Gene Therapy* 9:1885-1898, 1998). The Hep3B tumors were injected with  $1 \times 10^7$  PFU of AdLuc plus buffer, or  $1 \times 10^7$  PFU of AdLuc plus  $5 \times 10^7$  PFU of KD1, KD3, *d*/01/07, or *d*/309. After 2 weeks, mice were sacrificed and tumors excised. Proteins were extracted from the tumors and luciferase activity determined using a luminometer. The luciferase counts per tumor were 6,800 for AdLuc plus buffer, 113,500 for KD1, and 146,900 for KD3 (Fig. 11). Thus, KD3 and KD1 respectively caused a 22-fold and 17-fold increase in luciferase activity. This increase could be due to elevated synthesis of luciferase in cells that were initially coinfecting the AdLuc and KD1 or KD3, and it could also be due to spread of AdLuc from cell to cell in the tumor as mediated by KD1 or KD3.

In summary, infecting a tumor with a replication-competent ADP-overexpressing vector according to the invention together with a RD vector expressing an anti-cancer gene product should greatly increase the amount of anti-cancer protein synthesized in the tumor thereby increasing the ability of the replication-defective vector to promote destruction of the tumor.

#### Example 6

This example illustrates the construction and characterization of a recombinant Ad vector according to the invention which is replication-restricted to cancerous type II alveolar cells.

As demonstrated above, the *d*/01/07 mutation in KD1 and KD3 limits growth of these vectors to cancer cells. To further restrict their replication phenotype, the E4 promoter in each virus was deleted and replaced by the surfactant protein B (SPB) promoter to produce vectors named KD1-SPB (SEQ ID NO:14), KD3-SPB (SEQ ID NO:15), and *d*/01/07-SPB (SEQ ID NO:16). The SPB promoter is only active in cells containing the TTF1 transcription factor, which has thus far been found primarily in type II alveolar cells of the human lung (Lazzaro et al., *Development* 113:1093-1104, 1991). Thus, KD1-SPB, KD3-SPB, and *d*/01/07-SPB should be severely restricted to cancerous type II alveolar cells of the human lung. Many lung cancers are of this type.

The KD1-SPB and KD3-SPB vectors were prepared as follows. The E4 promoter is located at the right end of the Ad genome (Fig. 1). Using a pCRII-based plasmid (Invitrogen)

containing the Ad5 DNA sequences from the BamHI site (59 map units) to the right hand end of the genome, and using a PCR-based protocol, nearly all the transcription factor binding sites were deleted from the E4 promoter Ad5 base pairs 35,623 to 35,775 and replaced with a 500 base pair fragment containing the SPB promoter (Yan et al., *J. Biol. Chem.* 270:24852-24857, 1995). The final plasmids contain the E4-SPB substitution in the E4 region and the *d*/01/07, KD1, or KD3 versions of the E3 region, respectively, for the viruses *d*/01/07-SPB, KD1-SPB, and KD3-SPB. These plasmids were co-transfected into 293 cells with a fragment containing the left portion of the genome of *d*/01/07, and plaques were allowed to develop. Plaques were screened for the expected features, purified, then expanded into a stock.

10 The A549-TTF1 cell line was developed in order to test the prediction that replication of *d*/01/07-SPB, KD1-SPB, and KD3-SPB would be restricted to cancerous cells expressing the TTF1 transcription factor. These cells were co-transfected with two plasmids, one in which TTF1 is expressed from the CMV promoter, and the other coding for resistance to neomycin. Resistant clones were isolated and shown to express TTF1 activity as determined by transient transfection with a plasmid expressing chloramphenicol acetyltransferase from the TTF1-requiring surfactant protein C promoter.

KD1-SPB and KD1 were subjected to a standard plaque development assay on A549-TTF1 cells and parental A549 cells. The results are shown in Fig. 12. With KD1-SPB on A549 cells, plaques were not visible after 8 days, only about 4% of the final number of plaques were seen after 10 days, and about 50% of final plaques were seen after 12 days. With KD1-SPB on A549-TTF1 cells, plaques were visible after 6 days, and about 60% of plaques were seen after 10 days. Thus, as expected, KD1-SPB grew significantly faster on the cells containing TTF1. KD1 formed plaques more quickly than KD1-SPB on both A549 and A549-TTF1 cells, indicating that the E4 promoter-SPB substitution is not as effective the wild-type E4 promoter in inducing Ad replication. However, this difference between KD1-SPB and KD1 on A549-TTF1 cells is tolerable, with KD1-SPB delayed only about 1 day. Curiously, the final titer obtained for all virus stocks by day 16 was similar, indicating that A549 cells may contain a very small amount of endogenous TTF1 activity. It is predicted that KD3-SPB and *d*/01/07-SPB will behave similarly to KD1-SPB when grown in A549-TTF1 cells and A549 cells.

The restriction of KD1-SPB to cells containing TTF1 was further examined in a cell spread assay using H441 cells, a TTF1-expressing human pulmonary adenocarcinoma cell line (Yan et al., *supra*), and Hep3B cells, a liver cancer cell line not expected to express TTF1. Culture dish wells containing H441 or Hep3B cells were infected with KD1-SPB or KD1 at multiplicities ranging from 10 to 10<sup>-4</sup> PFU/cell. The H441 and Hep3B cells were



stained with crystal violet at 5 days and 8 days p.i., respectively. KD1-SPB and KD1 grew and spread equally well on H441 cells, causing destruction of the monolayer at  $10^{-1}$  PFU per cell (Fig. 13). (Some of the H441 monolayer has peeled off in the well with KD1-SPB at  $10^{-2}$  PFU per cell, and in the wells with KD1 and KD1-SPB at  $10^{-4}$  PFU per cell; this occasionally occurs in cell spread assays, and it does not reflect virus infection). With Hep3B cells, KD1 grew and spread very much better than KD1-SPB, with  $10^{-2}$  PFU per cell of KD1 causing more destruction of the monolayer as 1.0 PFU per cell of KD1-SPB (Fig. 13).

In summary, this example demonstrates that a replication-competent Ad, which replicates well on cells expressing the appropriate transcription factor, can be constructed with a tissue-specific promoter substituted in place of the E4 promoter. This methodology should be applicable to many other tissue specific and cell type specific promoters. One possibility would be a liver-specific promoter. Another possibility would be to use the E2F promoter, or another promoter with E2F sites, inasmuch as that promoter would be active only in cells such as cancer cells that have free E2F. A third possibility would be to use a regulatable promoter, e.g. the synthetic tetracycline response promoter (Massie et al., *J. Virol.* 72:2289-2296, 1998), where the activity of the promoter is controlled by the level of tetracycline or a tetracyclin analog in the patient.

#### Example 7

This example illustrates the construction and characterization of vectors which overexpress ADP and are not replication restricted.

As demonstrated above, the *d/01/07* E1A mutation in KD1 and KD3 is attenuating, inhibiting growth in non-dividing and even in dividing primary human epithelial and endothelial cells. Ads with this mutation are able to replicate well in dividing cancer cells. However, replication of such E1A mutants is not as efficient as, e.g. *d/309* which has a wild-type E1A gene. For instance, the rate of replication of *d/01/07*, as determined by the rate at which plaques develop, is reduced such that *d/01/07* plaques appear one day later than those of *d/309* (data not shown). This delay is due in part to a delay in expression of Ad late genes (see Fig. 3). The idea that the *d/01/07* mutation retards the rate of replication in A549 cells is further supported by the data in Fig. 8A, where *d/01/07* did not prevent tumor growth nearly as well as *d/309*. Despite this negative effect of the *d/01/07* E1A mutation, there are theoretical and practical aspects of having this mutation in the KD1 and KD3 vectors, as has been discussed. Nevertheless, one can easily imagine scenarios (e.g. patients with terminal cancer) where the ability of an Ad vector to destroy the tumor supercedes the requirement that the vector be totally restricted to tumor cells. In such cases, it would be advantageous to have vectors similar to KD1 and KD3, but with the wild-type E1A gene. The rates at which such

vectors express their genes, lyse cells, and spread from cell to cell should be higher than those of KD1 and KD3. Such vectors might cause some damage to non-cancerous cells and tissue, but this is also true for other modes of anti-cancer treatment such as surgery, chemotherapy, and radiation therapy.

5 In light of these considerations, vectors named GZ1 and GZ3 have been constructed that are identical to KD1 and KD3, respectively, except they have a wild-type E1A region. These vectors were constructed by overlap recombination in A549 cells. The left hand fragment contained the wild-type E1A region of Ad5, and the right end fragment contained the E3 modifications of KD1 or KD3. Plaques were picked, analyzed for the expected  
10 genotype, plaque-purified, and expanded into CsCl-banded stocks. The titers of these stocks on A549 cells were  $2.9 \times 10^{10}$  PFU/ml for GZ1 and  $1.6 \times 10^{11}$  PFU/ml for GZ3. Thus, these vectors can be grown into high titer stocks comparable to wild-type Ad. The GZ1 and GZ3 plaques are larger and appear much sooner than the plaques for *dl309*. Large rapidly-appearing plaques reflect the ability of Ad to lyse cells and spread from cell-to-cell (Tollefson  
15 et al., *J. Virol.* 70:2296-2306, 1996; Tollefson et al., *Virology* 220:152-162, 1996), and this property, as discussed, is due to the function of ADP.

The rate of plaque appearance can be quantitated in a plaque development assay (Tollefson et al., *supra*). Here, a typical plaque assay is performed, and the plaques observed on subsequent days of the assay are calculated as a percentage of the number of plaques  
20 observed at the end of the plaque assay. As shown in Fig. 14, after 4 days of plaque assay on A549 cells, GZ1 and GZ3 had 48% and 34%, respectively, of the final number of plaques, whereas *dl309* had only 1%. It is very unusual in Ad plaque assays in A549 cells for plaques to appear after only 4 days. These large plaques reflect the overexpression of ADP. These GZ1 and GZ3 plaques appear sooner than those of KD1 and KD3 (data not shown), no doubt  
25 because GZ1 and GZ3 replicate faster because they have a wild-type E1A region.

GZ1 and GZ3 lyse cells and spread from cell to cell much more effectively than *dl309*. At 6 days p.i. of A549 cells, approximately as much monolayer destruction was observed with GZ1 and GZ3 at  $10^{-3}$  PFU per cell as was observed with *dl309* at  $10^{-1}$  PFU per cell (Fig. 15, top panel). This result further underscores the conclusion that overexpression of  
30 ADP promotes cell lysis and virus spread.

In theory, GZ1 and GZ3 should be able to replicate not only in tumor cells but also in normal cells. Although they can replicate in normal cells, it is quite possible that GZ1 and GZ3 may be useful as anti-cancer vectors. First, GZ1 and GZ3 could be injected directly into the tumor. Many tumors are self-contained (encapsulated) except for the blood supply. The  
35 physical barriers of the tumor could minimize dissemination of the virus to other tissues.

Second, Ads are in general quite benign. Most infections of Ad5 are in infants and result in mild or asymptomatic disease, and are held in check by strong humoral and cellular immunity. Anti-Ad immunity appears to be life-long. GZ1 and GZ3 could be used only in patients who have an intact immune system, and perhaps also with pre-existing anti-Ad immunity. Further, patients could be passively immunized against Ad, using gamma-globulin or even specific purified anti-Ad neutralizing antibodies. Third, considering that Ad5 is a respiratory virus which most efficiently infects lung epithelial cells displaying the specific Ad5 receptor (named CAR) as well as specific integrins (e.g.  $\alpha_v\beta_5$ ), replication-competent vectors derived from Ad5 may not spread efficiently in many non-cancer tissues of the body. In addition, it is believed that versions of GZ1 and GZ3 can be constructed that have the E4 promoter substituted with a tumor-specific, tissue-specific, cell-specific, or synthetic promoter. Such vectors would have the positive features associated with wild-type E1A and ADP, and yet be replication-restricted to tumor tissue and/or to particular cell types.

#### Example 8

This example illustrates that the combination of KD1, KD3, GZ1, or GZ3 with radiation is more effective in destroying A549 cells, growing in culture or growing as tumors in nude mice, than the vectors alone or radiation alone.

This was shown in a cell spread assay. A549 cells growing in three 48 well culture dishes were mock-infected or infected with different viruses at multiplicities of infection ranging from 10 to  $10^{-4}$  PFU per cell as indicated in Fig. 15. One dish was not radiated. A second dish received 600 centigrays (cGy) of radiation at 24 h p.i., and a third dish received 2000 cGy of radiation at the same time. All dishes were stained with crystal violet at 6 days p.i. With the cells that were not radiated (top panel in Fig. 15), KD1 and KD3 caused monolayer destruction at lower multiplicities of infection than their parental control, *dI01/07*. This was also true for GZ1 and GZ3 as compared to their parental control *dI309*. (The paucity of cells in the cells infected with GZ1 or GZ3 at  $10^{-4}$  PFU per cell is an experimental artifact, and is not caused by infection by GZ1 or GZ3). These KD1, KD3, GZ1 and GZ3 results are consistent with earlier results showing that overexpression of ADP leads to increased cell lysis and virus spread.

With the dish that was infected then radiated with 600 cGy there was markedly increased cell killing and virus spread as compared to the non-radiated cells (compare the bottom panel of Fig. 15 with the top panel). For example, with KD1, KD3, GZ1, and GZ3 there was about the same amount of cell destruction in the radiated wells at  $10^{-4}$  PFU per cell as in the non-radiated wells at  $10^{-2}$  PFU per cell. Similar results were seen with the dish that

received 2000 cGy of radiation (data not shown), and also with dishes that received 600 or 2000 cGy of radiation 24 h prior to infection (data not shown).

The amount of cell destruction was quantitated by extracting the crystal violet from the cells with 33% acetic acid, then measuring the absorbance at 490 nm (data not shown).

- 5 The absorbance with non-radiated mock-infected cells was set at 100% cell viability. With mock-infected cells that received 600 cGy there was a 15% loss in viability (i.e. 15% less crystal violet was extracted). With KD1 at  $10^{-3}$  PFU per cell, the non-radiated cells were 80% viable whereas the cells receiving 600 cGy of radiation were only about 30% viable. Similar differences in viability between radiated and non-radiated cells were seen with KD3, GZ1, and GZ3. These results argue that the combination of radiation plus vector has a synergistic effect on cell lysis and vector spread, rather than an additive effect. If the effect were only additive, then with the KD1 samples at  $10^{-3}$  PFU per cell, the cell viability should have been 65% (15% reduction in viability due to radiation alone, 20% reduction due to KD1 alone). In fact, the cell viability was 30% rather than 65%.

- 15 As mentioned, approximately as much cell lysis and virus spread were observed with 600 cGy as with 2000 cGy. To determine the optimal dose of radiation to synergize with the vectors, an experiment similar to the one described above was conducted with mock-, dI01/07-, KD1-, KD3-, dI309, GZ1-, or GZ3-infected A549 cells. The 48 well plates received 0, 150, 300, or 600 cGy of radiation at 24 h p.i. Cells were stained with crystal violet. The results with cells receiving 0 versus 600 cGy of radiation were similar to those in Fig. 15. The crystal violet was extracted from the cells infected with  $10^{-3}$  PFU per cell of the difference viruses. The absorbance of crystal violet was determined, and the percent cell viability was graphed, using the absorbance of the non-radiated mock-infected cells as 100% cell viability. As illustrated in Fig. 16, an approximately linear decrease in cell viability in all wells was obtained with increasing radiation dose, although the slope of the line was more negative with KD1, KD3, GZ1, or GZ3 than with mock, dI01/07, or dI309. With KD1, KD3, GZ1, and GZ3, there was much more cell lysis and vector spread with their parental control viruses, and there was synergy between the vectors and radiation. For example, with mock-infected cells, 600 cGy reduced cell viability by about 30% (70% of cells were viable). KD1 without radiation reduced cell viability by about 23%. The combination of 600 cGy radiation plus KD1 reduced cell viability to about 85%, more than 53% of which is the sum of radiation alone and KD1 alone. When considering the data in Figs. 15 and 16 together, a dose of about 600 cGy is optimal in this type of cell culture experiment.

- 35 The combination of KD3 or GZ3 with radiation was also examined in the A549 tumor-nude mouse model (see Example 4). A549 cells were injected into the hind flanks of

nude mice, and tumors were allowed to form. When tumors reached approximately 50- $\mu$ l, they were injected with buffer or with  $5 \times 10^8$  PFU of KD3 or GZ3. Eight to ten tumors were injected per test condition. At 1 day p.i., half the mice received 600 cGy of whole body radiation. Tumor size was measured over time, and was plotted as a fold-increase in tumor size versus days p.i. (as described in Example 4). As shown in Fig. 17, the non-radiated buffer-injected tumors grew faster than those injected with KD3 or GZ3. Tumors that received the combination of KD3 and radiation did not grow, and those that received the combination of GZ3 and radiation shrank in size after 14 days. These results indicate that the combination of KD3 plus radiation or GZ3 plus radiation is more effective than either vector alone or radiation alone in reducing the rate of A549 tumor growth in nude mice. It is likely that radiation would increase the effectiveness in treating tumors of KD1 and GZ1, or indeed any other replication-competent or replication-defective Ad vector.

The mechanism by which radiation causes the ADP overexpressing vectors to lyse cells and spread from cell-to-cell more effectively is not understood. Radiation is expected to induce cellular DNA repair mechanisms, and that may allow for more efficient synthesis of Ad DNA. Radiation may enhance the function of ADP. ADP probably functions by interacting with one or more cellular proteins, and radiation may affect this protein(s) such that ADP functions more efficiently.

It is believed that KD1, KD3, GZ1, or GZ3, or any other replication-competent Ad vector, when used in combination with radiation, will be more effective than vector alone or radiation alone in providing clinical benefit to patients with cancer. The vectors should allow more tumor destruction with a given amount of radiation. Stated another way, radiation should cause more tumor destruction with a given amount of vector. These vectors should also allow the radiation oncologist to use less radiation to achieve the same amount of tumor destruction. Less radiation would reduce the side effects of the radiation.

It is also believed that a cocktail of vectors when used in combination with radiation will be more effective than the cocktail alone or radiation alone. The cocktail could consist of ADP producing vectors plus one or more replication defective vectors expressing an anticancer therapeutic protein (see Example 5).

#### Example 9

This example illustrates a structure-function analysis of adenovirus death protein.

ADP is an 11.6 kDa N-linked O-linked integral membrane glycoprotein that localizes to the inner nuclear membrane (NM) (Scaria et al., Virology 191:743-753). As illustrated in Fig. 18, the Ad2-encoded ADP (SEQ ID NO:6) consists of 101 amino acids; aa 1-40 (SEQ ID NO:17) are luminal, aa 41-59 (SEQ ID NO:18) constitute the transmembrane signal-anchor

(SA) domain, aa 63-70 (SEQ ID NO:19) constitute a basic proline (BP) domain within the nucleoplasmic (NP) domain, which constitutes aa 61-101 (SEQ ID NO:20). To determine which domains in ADP are required to promote cell death, a number of deletion mutants of *rec700* were prepared which lacked various portions of the ADP gene and examined for the ability of ADP to localize to the NM and promote death. The *rec700* virus is an Ad5-Ad-Ad5 recombinant, which has been described elsewhere (Wold et al., *Virology* 148:168-180, 1986).

The structure of ADP in *rec700* and in each deletion mutant is schematically illustrated in Fig. 18. The ADP gene in each deletion mutant has been sequenced using PCR methods to insure that the mutations are correct. The structure and activity of ADP in the deletion mutants was tested by infecting A549 cells followed by immunoblot analysis of the ADP mutant proteins as well as the ability to lyse cells. All deletion mutants expressed a stable ADP protein except *pm734.1* ( $\Delta 1-48$ , i.e. aa 1-48 are deleted). The *pm734.7* ( $N_{14}$ ) ADP, which has Asn<sub>14</sub> mutated to Ser, is O-glycosylated but not N-glycosylated because Asn<sub>14</sub> is the only N-glycosylation site (data not shown). The *dl735* ( $\Delta 4-11$ ) ADP is N-glycosylated but not O-glycosylated because the sites for O-glycosylation are deleted (data not shown). The *pm734.4* (M56) ADP, which has Met<sub>56</sub> in the SA domain mutated to Ser, contains exclusively N-linked high-mannose oligosaccharides (data not shown); this occurs because the Met<sub>56</sub> mutation precludes exit of ADP from the endoplasmic reticulum (ER). The *dl738* ADP, which lacks aa 46-60 in the signal-anchor domain, forms insoluble aggregates in the cytoplasm; therefore, aa 41-59 do in fact include the signal-anchor domain. The *pm734* ( $\Delta 1-40$ ) ADP, which initiates at Met<sub>41</sub> at the N-terminus of the SA domain, comigrated with the lower group of bands generated by proteolytic processing (data not shown). This indicates that the proteolytic cleavage sites occur near Met<sub>41</sub>. Consistent with this, the proteolytic products were not seen with *dl737* ( $\Delta 29-45$ ) (data not shown). Also, the size of the products decreased in all mutants with deletions within aa 41-101 (*dl715.1*, *dl715*, *dl714*, *dl716*) (data not shown).

The ability of these mutants to promote cell death was monitored by trypan blue exclusion, plaque development, and lactate dehydrogenase release assays (Tollefson et al., *J. Virol.* 70:2296-2306, 1996). The trypan blue results in Fig. 15A indicate that the death-promoting function of ADP was abolished by deletion of aa 1-40 (*pm734*), aa 11-26 (*dl736.1*), aa 18-22 (*dl735.1*), or aa 4-11 (*dl735*). Mutation of the N-glycosylation site at Asn<sub>14</sub> (*pm734.7*) reduced the death-promoting activity to about 50% of *rec700* (WT). *dl737* ( $\Delta 29-45$ ) was efficient as *rec700* in promoting cell death; this indicates that the proteolytic processing products must not be required to promote cell death because they are not formed with *dl737*. The SA domain is essential for death because *dl738* ( $\Delta 46-60$ ) and *pm734.4*

(M56) were completely defective (Fig. 19). *dl715.1* was nearly completely defective, indicating that the BP domain is extremely important. Surprisingly, aa 71-94 (*dl714*), 76-89 (*dl715*), and 79-101 (*dl716*) could be deleted without affecting the death-promoting activity of ADP (Fig. 19). On the other hand, deletion of aa 81-88 (*dl717*) nearly completely  
 5 abolished the activity of ADP (Fig. 19); this is probably the result of aberrant sorting of ADP (see below). Similar results were obtained when the ability of these ADP mutants to promote cell death was examined with standard plaque development, LDH-release and MTT assays.

The effects of these mutations on the intracellular localization of ADP are extremely interesting. When examined by immunofluorescence (IF) at 33 h p.i. (data not shown), ADP  
 10 from *rec700* (WT) localized crisply to the NM; localization to the Golgi was also apparent. With *dl714* ( $\Delta$ 71-94) and *dl715* ( $\Delta$ 76-89), ADP localized to all membranes, i.e. the ER, Golgi, plasma membrane, and NM. This was even more apparent at 45 h p.i. (data not shown). Thus, aa 71-94 appear to include a signal that directs ADP specifically to the NM. ADP is very likely sorted from the *trans*-Golgi network (TGN) to the NM, so this putative signal in  
 15 ADP probably functions in this sorting pathway. ADP from *dl717* ( $\Delta$ 81-88) is intriguing: it localized to the NM and Golgi, but in many cells "dots" and circular structures were observed. Again, this was more apparent at 45 h p.i. when these structures were the prominent feature. *dl717*-infected cells have not begun to die at 45 h p.i., so these structures are not cellular remnants. The intriguing possibility is that these structures are membrane vesicles that have  
 20 pinched off from the TGN but are defective in targeting to and/or fusing with the NM.

With *dl738* ( $\Delta$ 46-60 in the SA domain), ADP aggregated in the cytoplasm. This again indicates that aa 46-60 include the SA sequence. With *pm734.4* (M56), ADP localized primarily to the NM. As discussed above, the *pm734.4* ADP has exclusively high-mannose N-linked oligosaccharides, indicating that it never leaves the ER. Perhaps the putative NM-  
 25 localization signal in the C-terminal region of the *pm734.4* ADP targets ADP to the NM by lateral diffusion from the ER (which is continuous with the outer and inner NM).

With *dl737* ( $\Delta$ 29-45), ADP localized to the NM. ADP from *pm734* ( $\Delta$ 1-40), *pm734.7* (N14) (N-linked glycosylation cannot occur), and *dl735* ( $\Delta$ 4-11; the O-glycosylation sites are deleted) localized much more prominently to the Golgi than the NM. ADP from *dl735.1*  
 30 ( $\Delta$ 18-22) and *dl736.1* ( $\Delta$ 11-26) also localized much more strongly to the Golgi than the NM. Thus, residues 1-26 and/or glycosylation appear to be required for efficient transport of ADP from the Golgi/TGN to the NM.

In summary, aa 41-59 include the SA domain, Met<sub>56</sub> in the SA domain is required for exit from the ER, aa 1-26 are required for efficient exit from the Golgi, and aa 76-94 are  
 35 required to target ADP specifically to the NM. With respect to promoting cell death, the

essential regions are aa 1-26, the SA domain (ADP does not enter membranes), Met<sub>56</sub> in the SA domain, and the BP domain (aa 63-70). It is not clear whether the defective death-promoting phenotype of *pm734* ( $\Delta$ 1-40), *dI735* ( $\Delta$ 4-11), *dI735.1* ( $\Delta$ 18-22), *dI736.1* ( $\Delta$ 11-26), and *pm734.7* (N14) is due to lack of sequences (or oligosaccharides) that promote death or to much slower exit of ADP from the Golgi to the NM. *dI714* ( $\Delta$ 71-94) and *dI715* ( $\Delta$ 76-89) express a wild-type phenotype for promoting death even though they are defective in localizing specifically to the NM; this is probably because sufficient ADP still enters the NM to promote death. Even though the deletion in *dI717* ( $\Delta$ 81-88) lies within the deletions in *dI715* ( $\Delta$ 76-89) and *dI714* ( $\Delta$ 71-94), the *dI717* ADP is only about 15% as efficient as *rec700* (WT), *dI715* and *dI714* in promoting death. This may be because the *dI717* ADP tends to remain in vesicles rather than localizing to the NM. Altogether, these data indicate that ADP must localize to the NM in order to promote cell death.

#### Example 10

This example further characterizes the tissue specific Ad vectors described in Example 6. As discussed therein, the Ad E4 promoter is deleted and replaced with the promoter for surfactant protein B (SPB) in these vectors (Figure 24).

#### Materials and Methods

Cells, vectors and methods described in Example 6 were also used in this Example. In addition to the human cancer cell lines A549 (human lung carcinoma), Hep 3B (human hepatocellular carcinoma), and H441 (papillary lung adenocarcinoma) used in Example 6, HEK 293 cells (obtained from Microbix (Toronto, ON)) and VK10-9 cells were used. VK10-9 cells are 293 cells that in addition to E1 contain and express E4 and pIX. These cells will be referred to as 293-E4 cells.

Experiments employing phase contrast microscopy of Hep 3B and H441 cells were performed as follows. Monolayers of Hep 3B or H441 cells were grown in 60 mm dishes with 5 ml of DMEM (10% FBS), and were mock-infected or infected with KD1 or KD1-SPB at a multiplicity of infection of 10 plaque forming units (PFU) per cell. Phase contrast photographs of monolayers were taken at 4 and 7 days postinfection (p.i.).

Experiments employing western blots of H441 or Hep 3B cells were performed as follows. H441 or Hep 3B cells (in 60 mm dishes) were infected with 10 PFU/cell of KD1 or KD1-SPB. At 24 h p.i., the cells were washed three times with PBS and harvested by scraping. The cells were lysed by RIPA buffer. The protein concentration was measured by the BIO-RAD DC Protein Assay Kit (BIO-RAD Laboratories, Hercules, CA) and 10  $\mu$ g of each sample were electrophoresed on 15% sodium dodecylsulfate polyacrylamide gels (SDS-PAGE). The gels were electroblotted onto PVDF membranes (Immobilon, Millipore,



Bedford, MA). The membranes were blocked in TBST (50 mM Tris-Cl, pH 7.6, 150 mM NaCl, 0.2% Tween 20) containing 10% dry milk (Carnation) overnight at 4°C. After blocking, the membranes were incubated with a rabbit polyclonal antiserum against E4ORF3 (gift of Gary Ketner) or ADP (Tollefson et al., *J. Virol.* 66:3633-3642, 1992), or with M73, a  
5 monoclonal antibody against E1A (Harlow et al., *J. Virol.* 55:533-546, 1985). The secondary antibodies were goat anti-rabbit IgG-HRP or goat anti-mouse IgG-HRP. The blots were developed using the ECL protocol (Amersham Pharmacia, Arlington Heights, IL).

Experiments employing a lactate dehydrogenase release assay for cell lysis (Tollefson et al., *J. Virol.* 70:2296-2306) were performed as follows. H441 cells ( $7.7 \times 10^5$  cells per 35  
10 mm dish) and Hep 3B cells ( $9.0 \times 10^5$  cells per 35 mm dish) were infected at 20 PFU/cell in one ml serum-free DMEM. After an adsorption period of 1 h, 3 ml of DMEM (10% FBS) were added (final FBS concentration of 7.5%). Cells were incubated at 37°C with 6% CO<sub>2</sub>. At daily intervals, supernatants were collected, microfuged to remove floating cells, and cell-free supernatants were frozen at -70°C until assayed. Total lysis samples were prepared by  
15 addition of 10X lysis buffer included in the Cyto Tox 96 kit (Promega, Madison, WI). After all samples were collected, 20 µl samples were assayed in triplicate using the LDH assay kit Cyto Tox 96 and read on an EL340 Microplate reader (BioTec™ Instruments, Inc.) at 490 nm.

Experiments employing immunofluorescence evaluation of H441 and Hep 3B cells  
20 were performed as follows. H441 and Hep 3B cells were plated on Corning #1 coverslips in 35 mm dishes. H441 ( $1.5 \times 10^6$  cells/35 mm dish) and Hep 3B ( $9.0 \times 10^5$  cells/35 mm dish) were infected with 20 PFU/cell of the indicated viruses in 1 ml serum-free DMEM. After 1 h, 1 ml of DMEM/20% FBS was added (final concentration of 10% FBS). At the indicated times (48 h or 6 d p.i.), cells were fixed for 10 min in 3.7% paraformaldehyde in PBS, then  
25 permeabilized for 6 min in methanol (-20°C) and rehydrated in PBS. Coverslips were stained with rabbit anti-peptide antiserum against the Ad E2A-coded DNA binding protein (DBP) (1:400 dilution; gift of Maurice Green) and mouse monoclonal antibody against fiber (1:400 dilution; gift of Jeff Engler) or were stained with rabbit antiserum to E4ORF3 (1:250 dilution; gift of Gary Ketner). Secondary antibodies (Cappel/ICN) were used at 1:50 dilution. All  
30 antibodies were diluted in PBS containing 1% BSA and 0.1% sodium azide. Photographs were taken on a Nikon epifluorescence microscope using a 100X Planapo lens and Tmax 400 film (Kodak). The film was developed in Diafine developer.

Analysis of viral DNA replication by Southern hybridization was performed as follows. H441 and Hep 3B cells were grown in 60 mm dishes in DMEM supplemented with  
35 10% FBS. Cells were infected at 70% confluence with 10 PFU/cell of KD1 or KD1-SPB.

Dishes were incubated in humidified 5% CO<sub>2</sub> atmosphere at 37°C. Total genomic DNAs were isolated at 5, 24, 48, 72, and 96 h p.i. Equal amounts of total genomic DNAs were digested with HindIII and resolved on a 1% agarose gel prior to transfer onto membranes. A random primer <sup>32</sup>P-labeled pBHG10 plasmid probe (Bett et al., *Proc. Natl. Acad. Sci. USA* 91:8802-8806, 1994) was used for hybridization, and the blots were autoradiographed. DNA fragments were quantitated on a Molecular Dynamics PhosphorImager.

Virus yields were determined as follows. Hep 3B cells or H441 cells grown as monolayers in 35 mm dishes were infected with 10 PFU/cell of KD1 or KD1-SPB. At days 0 to 4 (for H441) or days 0 to 9 (for Hep 3B) p.i., cells and culture medium were frozen at -70°C. Samples were frozen and thawed three times to release the virus from the cells, and total virus yields were determined by plaque assay on A549 monolayers.

The effect of KD1-SPB and KD1 on H441 and Hep 3B tumors was examined in a nude mouse model (Doronin et al., *J. Virol.* 74:6147-6155, 2000). Tumor cells (10<sup>7</sup> cells in 200 µl of DMEM, 50% Matrigel [Becton Dickinson Labware, Bedford, MA] for H441 cells, or 10<sup>7</sup> cells in 200 µl of DMEM plus 10% Matrigel for Hep 3B cells) were injected into flanks of 5-6 weeks old athymic nude mice and allowed to grow for three weeks to about 100 µl (H441) or 150 µl (Hep 3B) volumes. Pre-established tumors (n = 10) were injected with 50 µl of DMEM or 5 x 10<sup>7</sup> PFU of indicated viruses in DMEM. Injections of the viruses were repeated twice weekly for 3 weeks to the total dose of 3.0 x 10<sup>8</sup> PFU per tumor. Tumor size measurements were taken twice per week for H441 cells, or weekly for Hep 3B cells using a Sylvac digital caliper. Tumor volumes were calculated in according to the formula: length x width<sup>2</sup> / 2. Data are represented as means of increase in tumor size relative to the tumor size at the initial injection.

### Results

The properties of KD1-SPB in various cell types were compared to those of its "parent", KD1. Figure 25 shows the plaque development properties of these vectors on 293-E4, 293, and A549 cells. The data are plotted as the number of plaques seen on any day of the plaque assay as a percentage of the number of plaques seen at the end of the assay (i.e. when new plaques cease to appear) (Tollefson et al., *J. Virol.* 70:2296-2306, 1966). This assay is an indicator of the size of the plaques. KD1 formed plaques equally well on 293-E4 and 293 cells (Figure 25A). With KD1-SPB, plaques were observed about 3-4 days sooner on 293-E4 compared to 293 cells (Fig. 2A). On A549 cells, KD1 formed plaques 4-6 days sooner than KD1-SPB (Figure 25B).

The properties of KD1-SPB versus KD1 were characterized in detail in H441 cells, a human papillary lung adenocarcinoma cell line known to express the TTF1 transcription

factor and in which the SPB promoter is active (Yan et al., *J. Biol. Chem.* 270:24852-24857, 1995). Hep 3B cells, a human hepatocellular carcinoma in which the SPB promoter should not be active, were used as a negative control. H441 and Hep 3B monolayers were infected with 10 PFU/cell of KD1 or KD1-SPB and photographed at 4 and 7 days p.i. Mock-infected  
5 Hep 3B cells formed a relatively homogeneous monolayer, but H441 cells tended to form structures that resemble syncytia (Figure 26A, B). As expected, KD1 produced cytopathic effect (CPE) on both cell lines at 4 and 7 days p.i. (Figure 26A, B). Also as expected, KD1-SPB caused CPE on H441 cells but not on Hep 3B cells. Since CPE in Ad-infected cells is usually an indicator of virus growth, these results suggest that KD1-SPB grows in H441 but  
10 not in Hep 3B cells.

To examine viral DNA replication, H441 and Hep 3B cells were infected with 10 PFU/cell of KD1 or KD1-SPB, then the accumulation of viral DNA was determined by DNA blot. With H441 cells, KD1 and KD1-SPB DNAs were readily detected at similar levels at 48-96 h p.i. (Figure 27A). With Hep 3B cells, KD1 DNA levels were similar to those in  
15 H441 cells, but KD1-SPB DNA was barely detectable. This was confirmed by PhosphorImager analysis of the DNA bands (Figure 27B).

Growth of KD1-SPB and KD1 in H441 and Hep 3B cells was determined by a single step growth assay. Cells were infected with 10 PFU/cell of vector, then total vector yield was determined by plaque assay. Total yield of both vectors was similar in H441 cells, reaching a  
20 plateau after 2 days (Fig. 28A). KD1 yield plateaued in Hep 3B cells after 2-4 days p.i. (Figure 28B). However, KD1-SPB levels were about 5 logs lower in Hep 3B cells after 2-4 days, and even by 9 days they had not achieved the levels of KD1. We conclude that KD1-SPB grows with significant specificity on H441 versus Hep 3B cells. Further, KD1-SPB grows as well as KD1 on H441 cells, indicating that the E4 promoter deletion by itself does  
25 not significantly compromise the vector, and that the E4 promoter can be replaced by a tissue-specific promoter in a replication-competent vector.

To obtain further details on the replication of KD1-SPB vs KD1 in H441 and Hep 3B cells, the expression of representative Ad proteins by KD1-SPB and KD1 was examined. H441 or Hep 3B cells were mock-infected or infected with 10 PFU/ml of KD1 or KD1-SPB,  
30 then at 24 h p.i. the proteins were extracted and the E1A, E4ORF3, and ADP proteins were examined by immunoblot. E4ORF3 is one of the six proteins coded by the E4 transcription unit (Leppard, *J. Gen. Virol.* 78:2131-2138, 1997). As anticipated, KD1-SPB expressed E4ORF3 well in H441 cells, but only at trace levels in Hep 3B cells (Figure 29). KD1-SPB expressed the E1A proteins in Hep 3B cells. Synthesis of E1A proteins by KD1-SPB in Hep  
35 3B cells is expected because E1A expression does not require E4 proteins; it also indicates

that the block to infection with KD1-SPB is downstream of E1A. KD1 expressed E1A in both cell lines, but the amount was less than obtained with KD1-SPB in Hep 3B cells (Figure 29). The increased E1A levels seen with KD1-SPB may reflect its poor ability to enter the late phase of infection (see Discussion). KD1-SPB expressed ADP as well as KD1 in H441  
5 cells, but it did not make detectable ADP in Hep 3B cells. ADP is primarily a late protein, so this result is consistent with the relative lack of E4 protein expression, DNA replication, and growth of KD1-SPB in Hep 3B cells.

To gain insights into replication events that occur in individual cells, expression of E4ORF3, the E2A-DBP, and the fiber late protein was examined by immunofluorescence.  
10 H441 or Hep 3B cells were infected with 20 PFU/cell. At 48 h or 6 days p.i., cells were fixed and immunostained. E4ORF3 was detected in the nuclei of H441 cells at 48 h p.i. with KD1, KD1-SPB, or dl309 (Figure 30A). (dl309 is an Ad5 mutant that has wild-type E1A, expresses Ad5 levels of ADP, and lacks the E3-RID and E3-14.7K genes). E4ORF3 could not be detected in the vast majority of Hep 3B cells infected with KD1-SPB (Figure 30A), even at 6  
15 days p.i. (Figure 30B). Thus, KD1-SPB expresses E4ORF3 well in H441 but not in Hep 3B cells.

Figure 31A shows double label immunofluorescence of DBP and fiber in the same Hep 3B cells at 48 h p.i. with KD1 or KD1-SPB. With KD1, there was a strong speckled staining pattern in the nucleus that is typical for DBP at 48 h p.i. (Figure 31A, top left panel).  
20 There was strong staining of fiber throughout these same cells (Figure 31A, top right panel). Staining of the cytoplasm and nucleus is expected because fiber is synthesized in the cytoplasm and then transported to the nucleus where virions assemble. With KD1-SPB at 48 h p.i., about 25% of the cells showed the speckled staining for DBP, and only one cell (7% of total) with the advanced speckled pattern was also stained for fiber (Figure 31A, bottom two  
25 panels). Even at 6 days p.i., only about 30% of cells showed staining for DBP, and about 20% for fiber (Figure 31B). Thus, markedly fewer Hep 3B cells infected with KD1-SPB expressed DBP and especially fiber as compared to KD1. These results indicate that KD1-SPB replicates as well as KD1 in H441 cells, no doubt because the SPB promoter is active in H441 cells (Yan et al., *J. Biol. Chem.* 270:24852-24857, 1995). KD1-SPB barely replicates  
30 in Hep 3B cells, presumably because the SPB promoter is minimally active in these cells.

At the culmination of replication, Ad-infected cells are lysed and the virus spreads to other cells; this process is mediated in large part by ADP (Tollefson et al., *Virology* 220:152-162, 1996; Tollefson et al., *J. Virol.* 70:2296-2306, 1996). To examine vector-induced cell  
35 lysis, H441 and Hep 3B cells were mock-infected or infected with 20 PFU/cell of KD1, KD1-SPB, or dl309, and cell lysis was determined by release of lactate dehydrogenase (Tollefson et

al., *J. Virol.* 70:2296-2306, 1996). All vectors lysed H441 cells beginning at 2-3 days p.i. (Figure 32A). KD1 and dl309 also lysed Hep 3B cells in the same time period; however, KD1-SPB caused only minimal cell lysis (Figure 9B). Thus, these data, along with the cell spread data in Example 6 and Figure 13, demonstrate that KD1-SPB lyses cells and spreads efficiently from cell-to-cell in H441 but not Hep 3B cells.

An experiment was conducted to determine whether KD1-SPB or KD1 would suppress H441 tumors in nude mice. H441 cells were injected into each hind flank. When tumors had grown to about 100  $\mu$ l (H441) or 150  $\mu$ l (Hep 3B), they were injected twice weekly for 3 weeks with DMEM (mock) or  $5 \times 10^7$  PFU of test virus in 50  $\mu$ l of DMEM ( $3.0 \times 10^8$  total PFU). Ten tumors (5 mice) were used for each virus. Growth of H441 tumors was suppressed similarly by KD1-SPB and KD1 (Figure 33A). KD1 suppressed growth of Hep 3B tumors, whereas KD1-SPB caused only minimal suppression (Figure 33B). These results show that KD1-SPB is as effective as KD1 in suppressing tumors when the SPB promoter is active. Further, the cell type specificity observed with KD1-SPB in vitro is maintained in vivo.

#### Discussion

Tumor specificity is one of the biggest challenges facing cancer gene therapy, i.e. having the therapeutic gene be expressed specifically in cancer cells. Specificity is very important for RC viruses. Two main strategies have been described that in theory confer specificity: transductional targeting and transcriptional targeting. Directing specificity of vectors toward specific cell surface receptors on the target cells has been attempted through various methods. Although this approach is theoretically attractive it might encounter multiple obstacles such as the lack of incorporation of the engineered protein into the virion (Scaria et al., *Virology* 191:743-753, 1992) or lack of infectivity through the targeted receptor (Cosset et al., *J. Virol.* 69:6314-6322, 1995). Transcriptional targeting utilizes tumor and tissue specific promoters. In replication-defective vectors these regulatory sequences confine the expression of cytotoxic genes to specific tissues. In replication-competent vectors, as an added layer of regulation, vector replication per se can be placed under the control of tumor or tissue specific promoter/enhancer sequences. In replication-competent Ad, insertion of the tissue or tumor specific promoter/enhancer into the E1A promoter/enhancer region has been used exclusively (Hallenbeck et al., *Hum. Gene Ther.* 10:1721-1733, 1999; Rodriguez et al., *Cancer Res.* 57:2559-2563, 1997; Yu et al., *Cancer Res.* 59, 4200-4203, 1999; Yu et al., *Cancer Res.* 59:1498-1504, 1999). The rationale behind these vectors is that expression of E1A and therefore the whole Ad transcription program will depend on these tissue or tumor specific promoters. However, as a generic approach, there may be difficulties. The E1A

enhancer/promoter is very complex. The enhancer controls not only the E1A promoter but also distant promoters such as the E4 promoter (Shenk, T. pp. 2111-2148 *In* B.N. Fields, D.M. Knipe, and P.M. Howley (eds.), *Fields Virology*, Lippincott-Raven, Philadelphia, 1996). In addition, it has been shown that the E1A enhancer in the inverted terminal repeat  
5 region changes tissue specificity of cellular promoters (Shi et al., *Hum. Gene Ther.* 8:403-410, 1997). Also, the E1A enhancer/promoter is partially embedded within the signals required to package the Ad genome into virions, and it may be problematic to remove all the E1A enhancer elements without impairing virus production. Accordingly, we chose to replace the E4 promoter with a tissue specific promoter. E4 genes are essential for Ad  
10 replication, and therefore we expected that the replication of the recombinant virus would be dependent on the tissue specific regulatory elements.

To construct KD1-SPB, the ca. 300 bp of the E4 promoter was deleted and the B-500 version (ca. 500 bp) of SPB promoter was inserted (Yan et al., *supra*) (Figure 24 C, D). We selected the SPB promoter because of its strict tissue specificity: it is exclusively active in  
15 type II alveolar cells and bronchial epithelial cells of the lung (Bohinski et al., 1994, *Mol. Cell. Biol.* 14:5671-5681, 1994). Since the parental virus KD1 contains and expresses two E1A mutations that restrict virus replication to tumor cells (Doronin et al., *supra*), we anticipated that the virus would selectively replicate in cells derived from lung tumors. Thus, H441 cells, a papillary lung carcinoma cell line, were used to characterize the replication,  
20 gene expression, and functional profile of KD1-SPB.

KD1-SPB formed plaques 3-4 days sooner on 293-E4 cells that express E4 proteins than on 293 cells, whereas KD1 formed plaques with the same kinetics on both cell lines. These data show that the E4 promoter is active in 293 cells, and that the SPB promoter displays very low activity in 293 cells. It is not clear why KD1-SPB forms plaques on 293  
25 cells; these cells are derived from human embryonic kidney and at least one of the transcription factors regulating the SPB promoter (Bohinski et al., *supra*), hepatocyte nuclear factor 3, is expressed in embryonic kidney. It is also possible that TTF1, the master regulatory factor of SPB expression, is minimally active in 293 cells.

KD1 grew to equally high titers in H441 and Hep 3B cells (Figure 28A, B). In  
30 contrast, KD1-SPB replicated as efficiently as KD1 in H441 cells, in which the SPB promoter is active (Yan et al., *supra*) (Figure 28A), but replicated poorly in Hep 3B cells, most likely because the SPB promoter is inactive (Figure 28B). This selectivity has been confirmed by measuring viral DNA production in the two cell lines. KD1-SPB DNA replication was similar both kinetically and quantitatively to KD1 DNA replication in H441, however in Hep

3B cells, KD1-SPB DNA was almost undetectable (Figure 27A, B). The cytopathic effect, a surrogate marker of Ad replication, showed a similar specificity (Figure 26).

To further confirm our predictions on the molecular basis of the observed tissue specificity we monitored viral protein expression. When cells were infected with KD1-SPB all the viral proteins early or late, except for E1A, were expressed in a tissue-specific fashion (high expression in H441, low to undetectable expression in Hep 3B) (Figures 29-31). We found a good correlation between the levels of E4 promoter activity (E4ORF3 expression) and the expression of E2A-DBP, ADP, and fiber proteins. Thus, the SPB promoter retains its tissue specificity in the Ad genome and it seems to be the limiting factor of Ad gene expression in the cell lines tested. As expected, expression of E1A is not tissue-specific. Thus, the regulatory step of tissue-specific Ad DNA replication is downstream of E1A. In Hep 3B cells, KD1-SPB expressed E1A at a higher level than did KD1 (Figure 29), strongly suggesting that KD1-SPB replication in most of the Hep3B cells remains at the early stage.

The cytolytic effect of KD1-SPB also showed a tissue-specific profile (Figure 32; Figure 13 of Example 6), i.e., preferential lysis of H441 cells over Hep 3B cells, a pattern similar to the specificity observed at the level of DNA replication (Figure 27) and viral protein synthesis (Figures 29-31). This cell type specificity was also observed when these cells were growing as tumors in nude mice. Growth of H441 tumors was suppressed by KD1-SPB and KD1 at similar efficacy (Figure 33A). In contrast, KD1-SPB unlike KD1 had only minimal effect on the growth of Hep 3B tumors (Figure 33B).

In summary, substitution of the E4 promoter with a tissue specific promoter allows highly tissue specific replication of Ad vectors and in the target tissue it is as efficient as the replication of the parental virus. KD1-SPB lacks all E3 genes except ADP. E3 gp19K, RID and 14.7K have been shown to protect Ad-infected cells from attack by cytotoxic lymphocytes and apoptosis-inducing cytokines such as tumor necrosis factor and Fas ligand (Wold et al., pp. 200-232 *In* A.J. Cann (ed.), *DNA Virus Replication: Frontiers in Molecular Biology*, Oxford University Press, Oxford, 2000; Wold et al., *Curr. Opin. Immunol.* 11:380-386, 1999).

The therapeutic index (virus produced in H441 cells compared to Hep 3B cells) of KD1-SPB is  $10^4$ - $10^5$  for the first 4-5 days (Figure 28). These data compare to data reported by Calydon ( $10^4$ - $10^5$ ) for their prostate specific viruses (Rodriguez et al., *supra*; Yu et al., *Cancer Res.* 59, 4200-4203, 1999; Yu et al., *Cancer Res.* 59:1498-1504, 1999). We suggest that KD1-SPB has some added advantage over vectors reported by other laboratories because it encodes a mutant form of E1A that restricts replication to cancer cells (Doronin et al., *supra*).

Although the lung ranks as the second highest cancer site for both men and women in the U.S. Reis et al., *Cancer Res.* 88:2398-2424, 2000), lung cancer has not been a major target for cancer vector gene therapy since intratumoral injection of virus is generally not feasible in the lungs. However, there has been a recent report of intratumor injection of a replication-  
5 defective Ad vector into a lung tumor, and such an approach could be attempted with KD1-SPB. It may also be feasible to administer KD1-SPB systemically in the lung.

In view of the above, it will be seen that the several advantages of the invention are achieved and other advantageous results attained.

As various changes could be made in the above methods and compositions  
10 without departing from the scope of the invention, it is intended that all matter contained in the above description and shown in the accompanying drawings shall be interpreted as illustrative and not in a limiting sense.

All references cited in this specification, including patents and patent  
applications, are hereby incorporated by reference. The discussion of references herein is  
15 intended merely to summarize the assertions made by their authors and no admission is made that any reference constitutes prior art. Applicants reserve the right to challenge the accuracy and pertinence of the cited references.



What is Claimed Is:

1. A recombinant vector which is replication-competent in a neoplastic cell and which overexpresses an adenovirus death protein.
2. The recombinant vector of claim 1 wherein the adenovirus death protein comprises amino acids 1-26, 41-59, and 63-70 of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, or SEQ ID NO:8 or a conservatively substituted variant thereof or wherein the adenovirus death protein comprises SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, or SEQ ID NO:8.
3. The recombinant vector of claim 2 which comprises a recombinant virus.
4. The recombinant vector of claim 3, wherein the recombinant virus is an adenovirus lacking expression of at least one E3 protein selected from the group consisting of: gp19K; RID $\alpha$ ; RID $\beta$  and 14.7K.
5. The recombinant vector of claim 4 which comprises SEQ ID NO:3 or SEQ ID NO:4.
6. The recombinant vector of claim 3 which is replication-restricted to neoplastic cells.
7. The recombinant vector of claim 6 which comprises SEQ ID NO:1 or SEQ ID NO:2.
8. The recombinant vector of claim 3, wherein the recombinant adenovirus comprises a tissue specific promoter, a tumor specific promoter, or an inducible promoter substituted for the E4 promoter.
9. The recombinant vector of claim 8, wherein the tissue-specific promoter is a surfactant protein B promoter.
10. The recombinant vector of claim 6 which comprises SEQ ID NO:14, SEQ ID NO:15 or SEQ ID NO:16.
11. The recombinant vector of claim 1, wherein the vector further comprises a gene encoding an anti-cancer product.
12. The recombinant vector of claim 11, wherein the gene encoding an anti-cancer product is in the E3 region of the vector.
13. A method for promoting death of a neoplastic cell comprising contacting the neoplastic cell with at least one vector which is replication competent in the neoplastic cell and which overexpresses an adenovirus death protein.
14. The method of claim 13 wherein the adenovirus death protein comprises amino acids 1-26, 41-59, and 63-70 of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, or SEQ

ID NO:8 or a conservatively substituted variant thereof or wherein the adenovirus death protein comprises SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, or SEQ ID NO:8.

15. The method of claim 14, wherein the vector comprises a recombinant adenovirus lacking expression of at least one E3 protein selected from the group consisting of: gp19K; RID $\alpha$ ; RID $\beta$  and 14.7K.

16. The method of claim 15, wherein the neoplastic cell comprises a tumor in a patient and the contacting step comprises administering the recombinant adenovirus to the tumor.

17. The method of claim 16, further comprising the step of passively immunizing the patient against the recombinant adenovirus.

18. The method of claim 17, wherein the recombinant adenovirus comprises SEQ ID NO:3 or SEQ ID NO:4.

19. The method of claim 15, wherein the vector is replication-restricted to neoplastic cells.

20. The method of claim 19, wherein the vector is a recombinant adenovirus comprising SEQ ID NO:1 or SEQ ID NO:2.

21. The method of claim 15, wherein the recombinant adenovirus comprises a tissue specific promoter or an inducible promoter substituted for the E4 promoter.

22. The method of claim 21, wherein the tissue specific promoter is a surfactant protein B promoter.

23. The method of claim 22, wherein the recombinant adenovirus comprises SEQ ID NO:14, SEQ ID NO:15 or SEQ ID NO:16.

24. The method of claim 16, further comprising treating the tumor with radiation.

25. The method of claim 24, comprising administering more than one recombinant adenovirus to the tumor and treating the tumor with radiation.

26. The method of claim 16, further comprising treating the tumor with chemotherapy.

27. The method of claim 26, comprising administering more than one recombinant adenovirus to the tumor and treating the tumor with chemotherapy.

28. The method of claim 16, further comprising administering to the tumor one or more replication-defective adenovirus which expresses an anti-cancer gene product, wherein the recombinant adenovirus complements spread of the replication-defective adenovirus in the tumor.

29. A composition comprising:

a first recombinant virus which is replication competent in a neoplastic cell and overexpresses an adenovirus death protein; and

a second recombinant virus which is replication defective and which expresses an anti-cancer gene product,

wherein the first recombinant virus complements replication of the second recombinant virus.

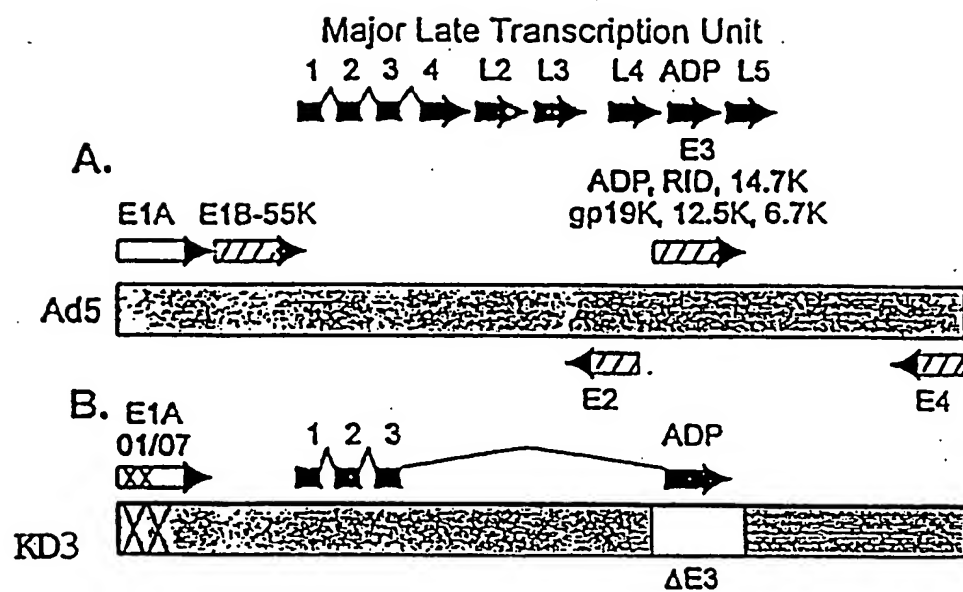
30. The composition of claim 29 wherein the first recombinant virus comprises a recombinant adenovirus lacking expression of at least one E3 protein selected from the group consisting of: gp19K; RID $\alpha$ ; RID $\beta$  and 14.7K.

31. The composition of claim 30 wherein the recombinant adenovirus comprises a nucleotide sequence selected from the group consisting of: SEQ ID NO:1; SEQ ID NO:2; SEQ ID NO:14; SEQ ID NO:15; SEQ ID NO:16; SEQ ID NO:3; or SEQ ID NO:4.

32. A composition comprising

a first recombinant virus which is replication-defective in a neoplastic cell and which overexpresses an adenovirus death protein, and

a second recombinant virus which is replication-competent in a neoplastic cell.



# ADP Is Expressed Earlier in Infection By KD1, KD3, GZ1, and GZ3

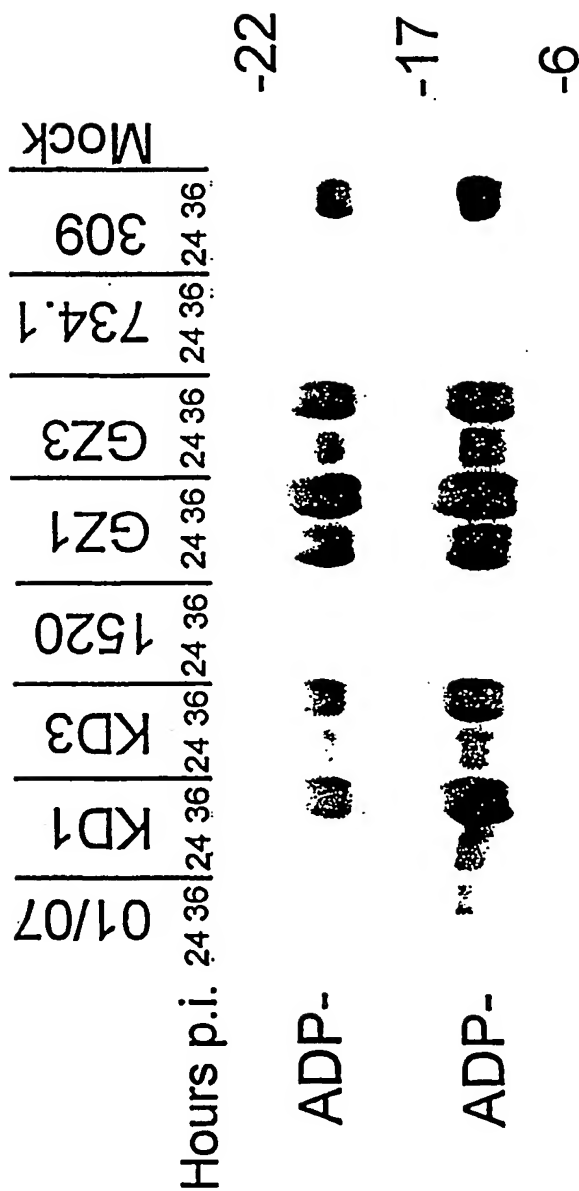


FIGURE 2

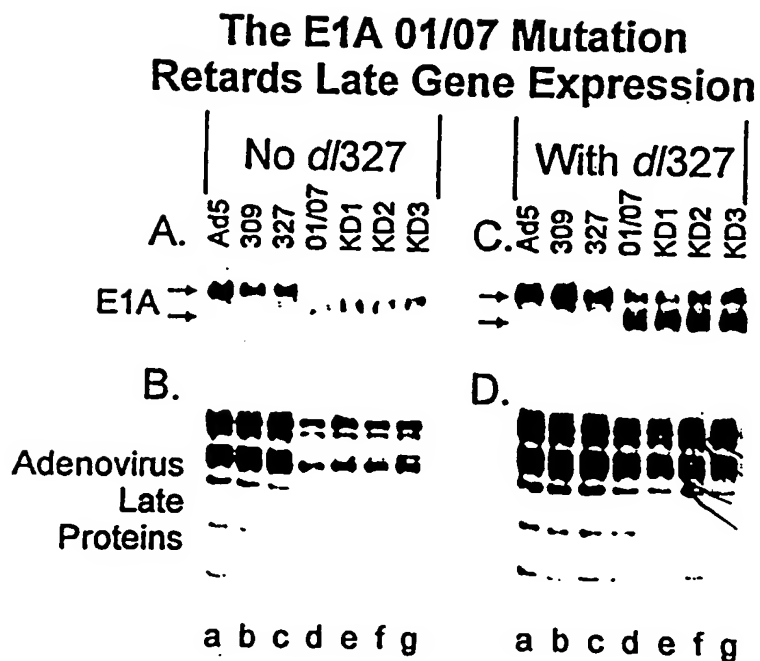


FIGURE 3

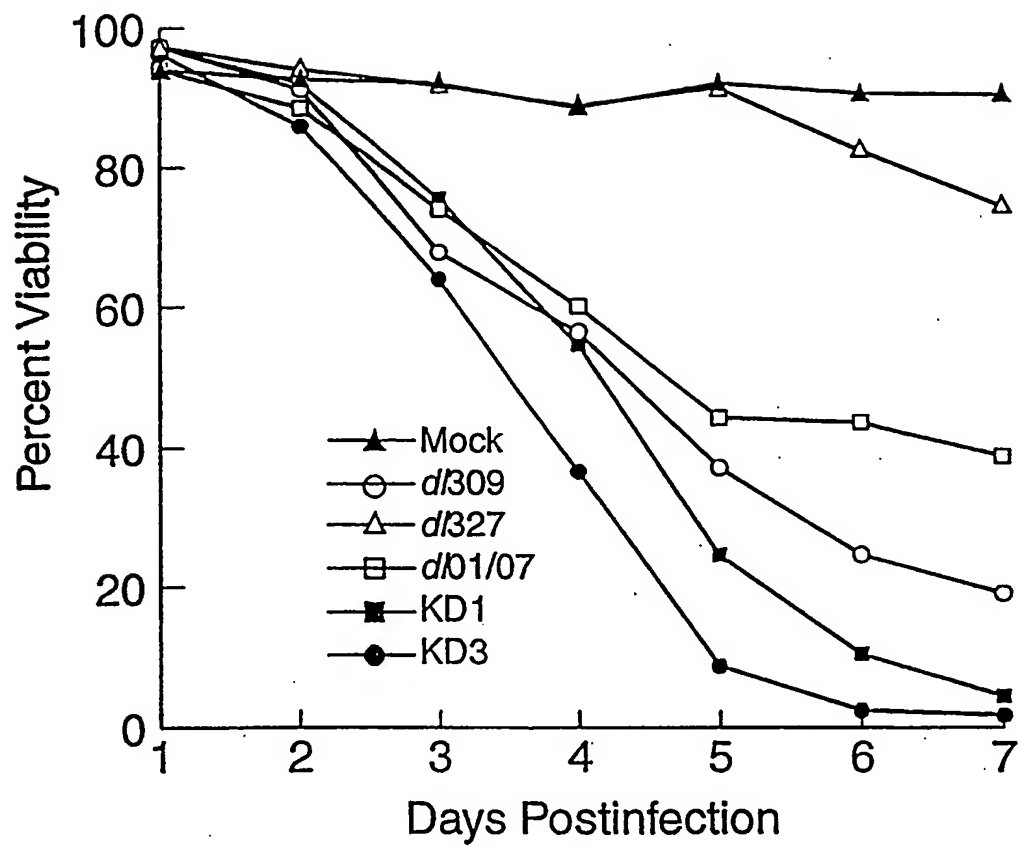


FIGURE 4

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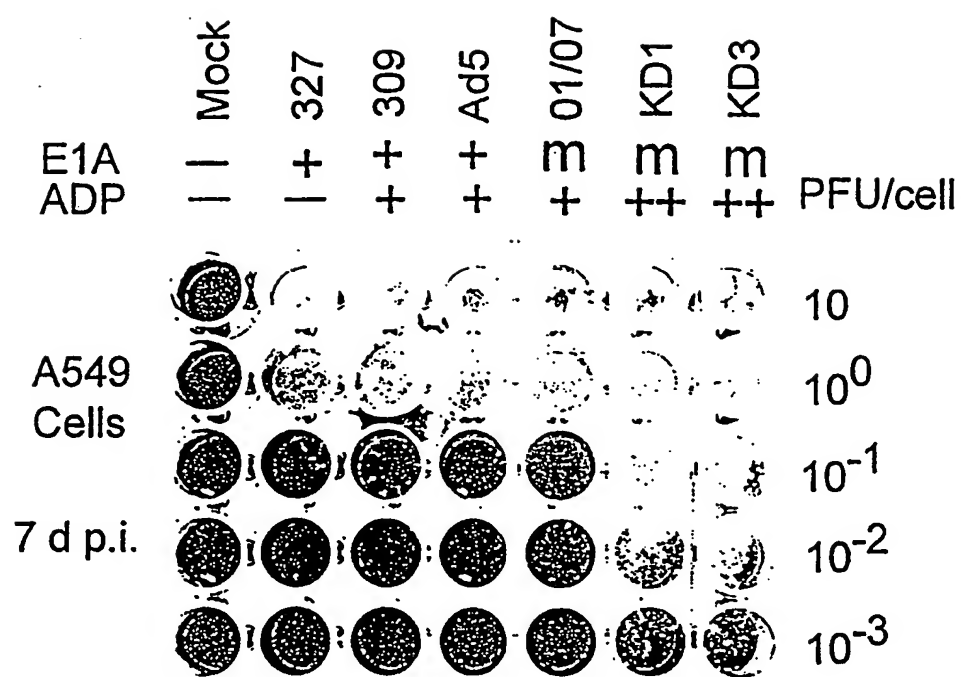


FIGURE 5

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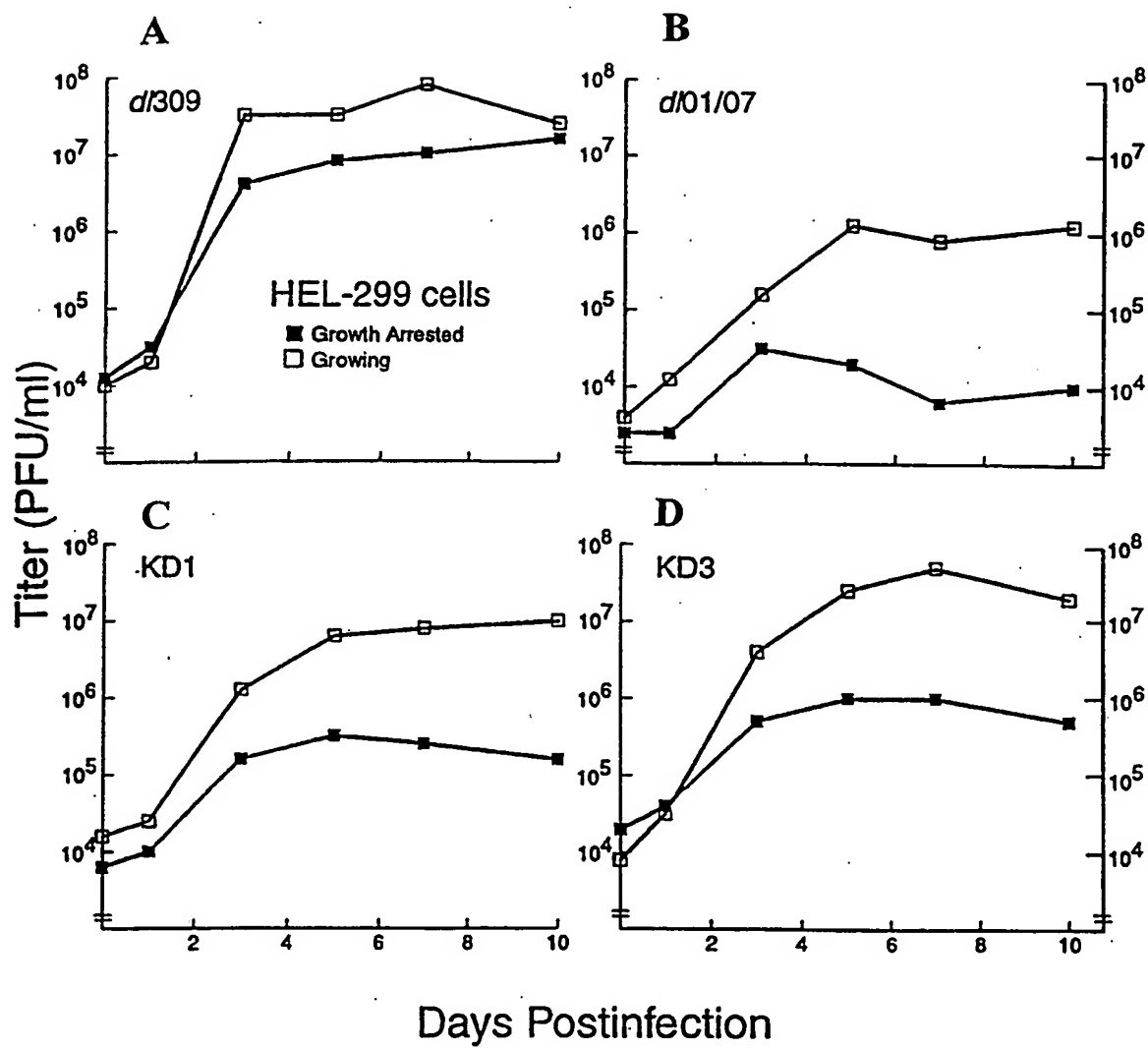


FIGURE 6

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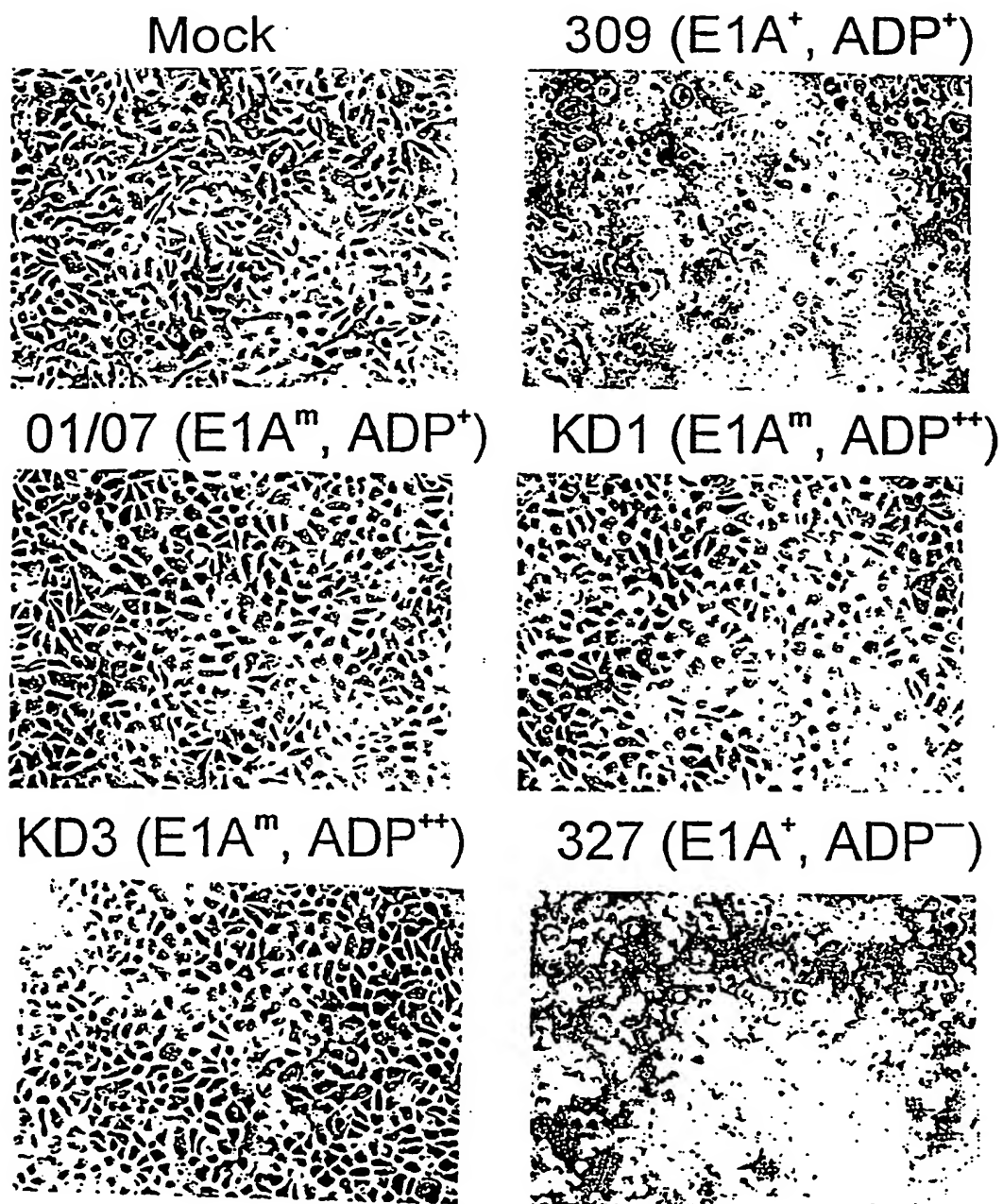


FIGURE 7

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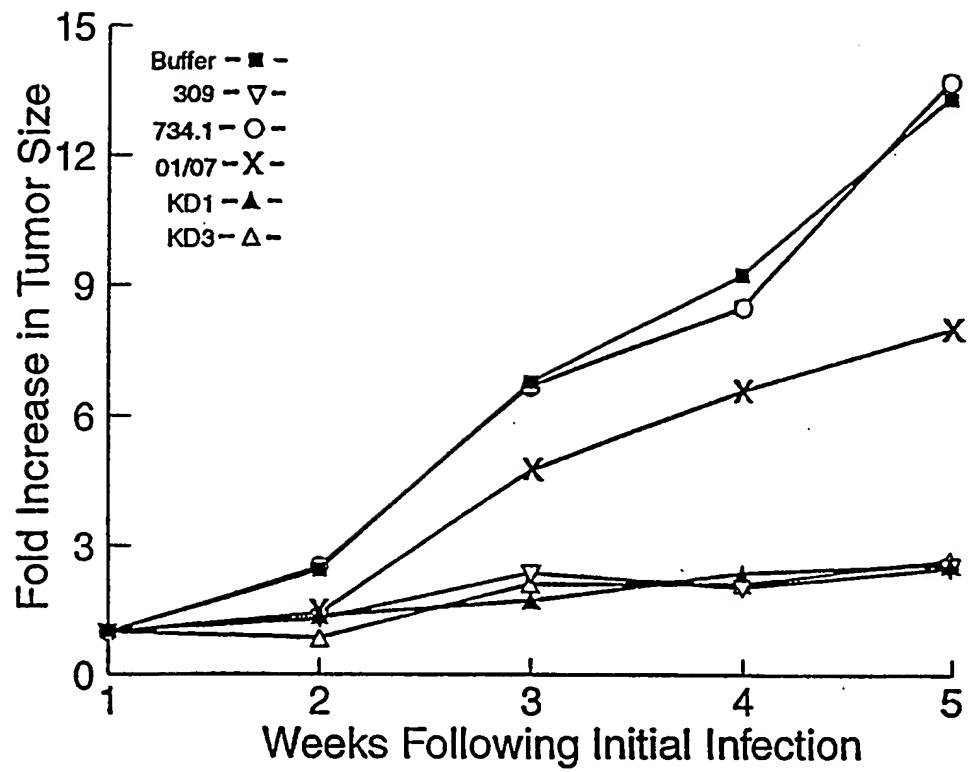


FIGURE 8A

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One Injection of KD3 or GZ3 Inhibits  
Growth of A549 tumors  
( $5 \times 10^8$  PFU injected on day 0)

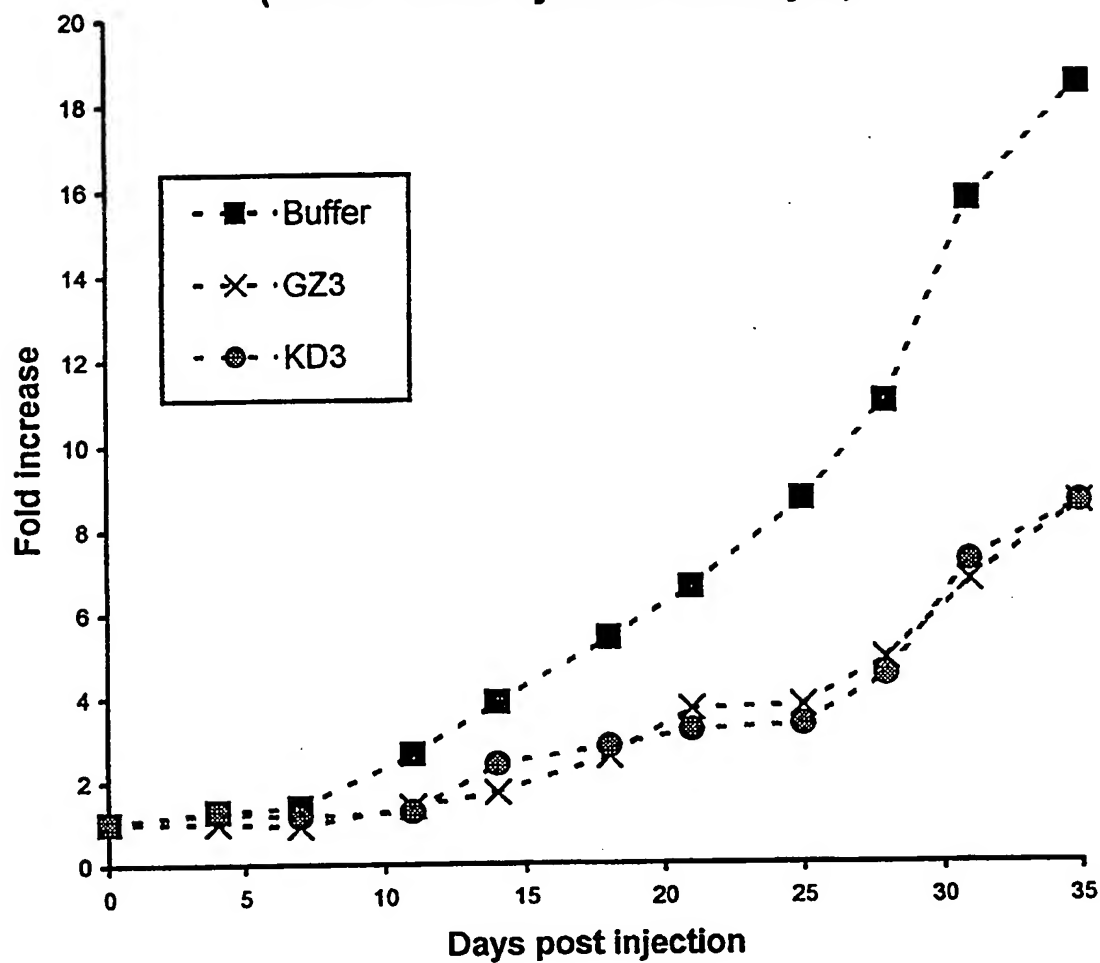


FIGURE 8B

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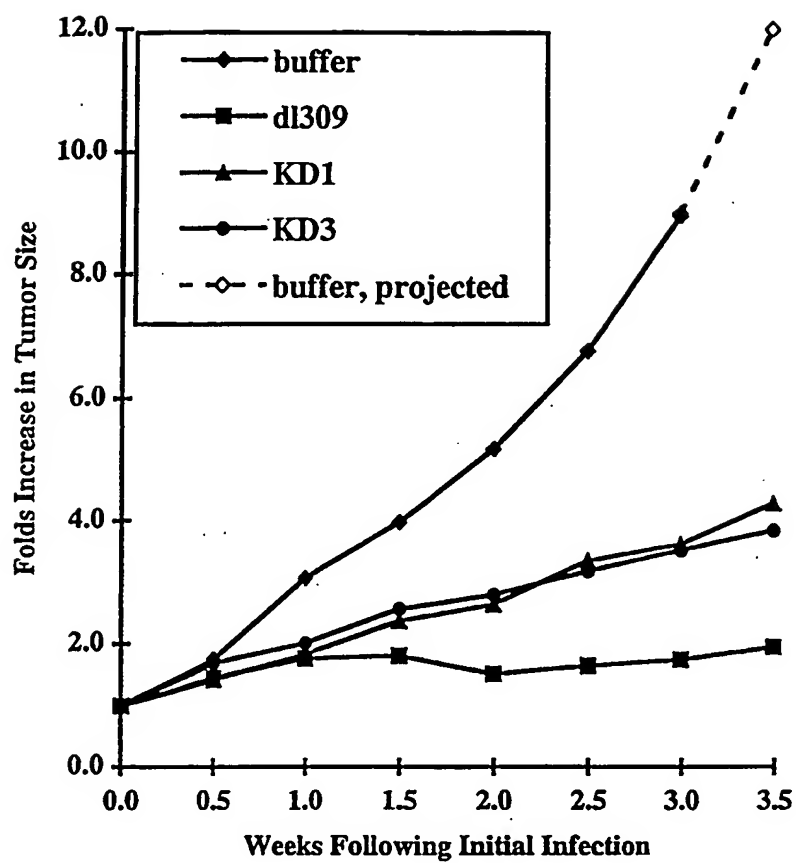


FIGURE 9

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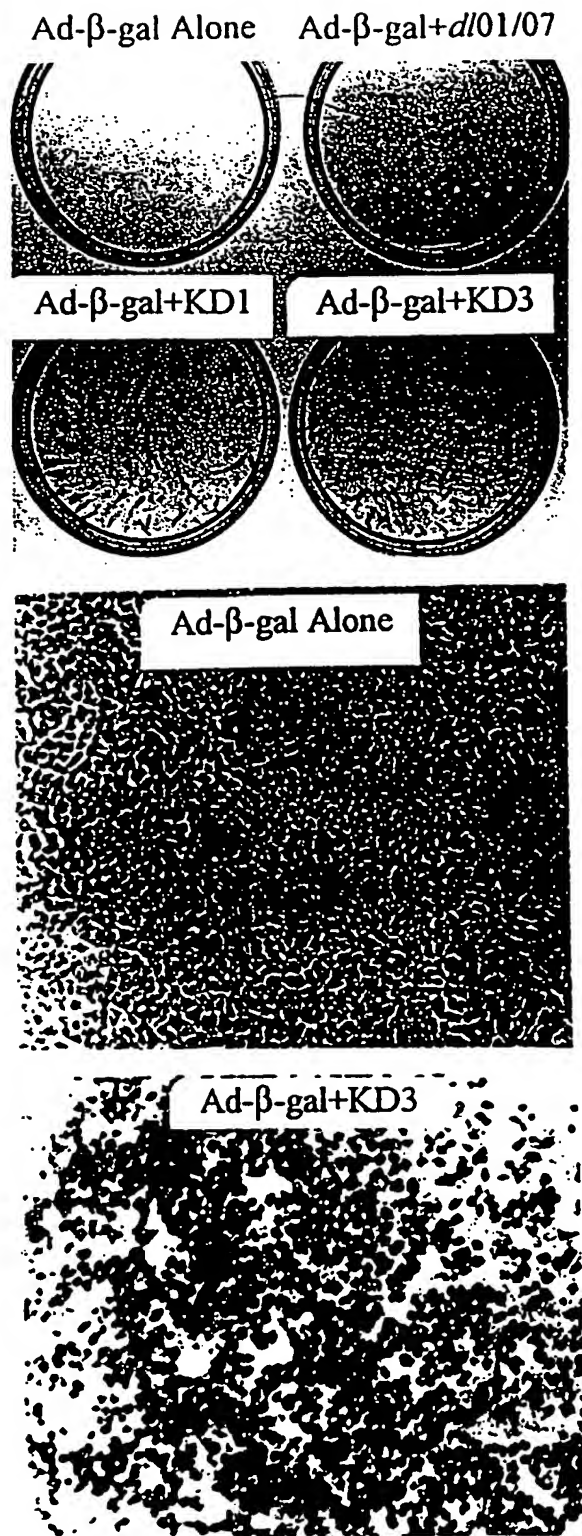


FIGURE 10

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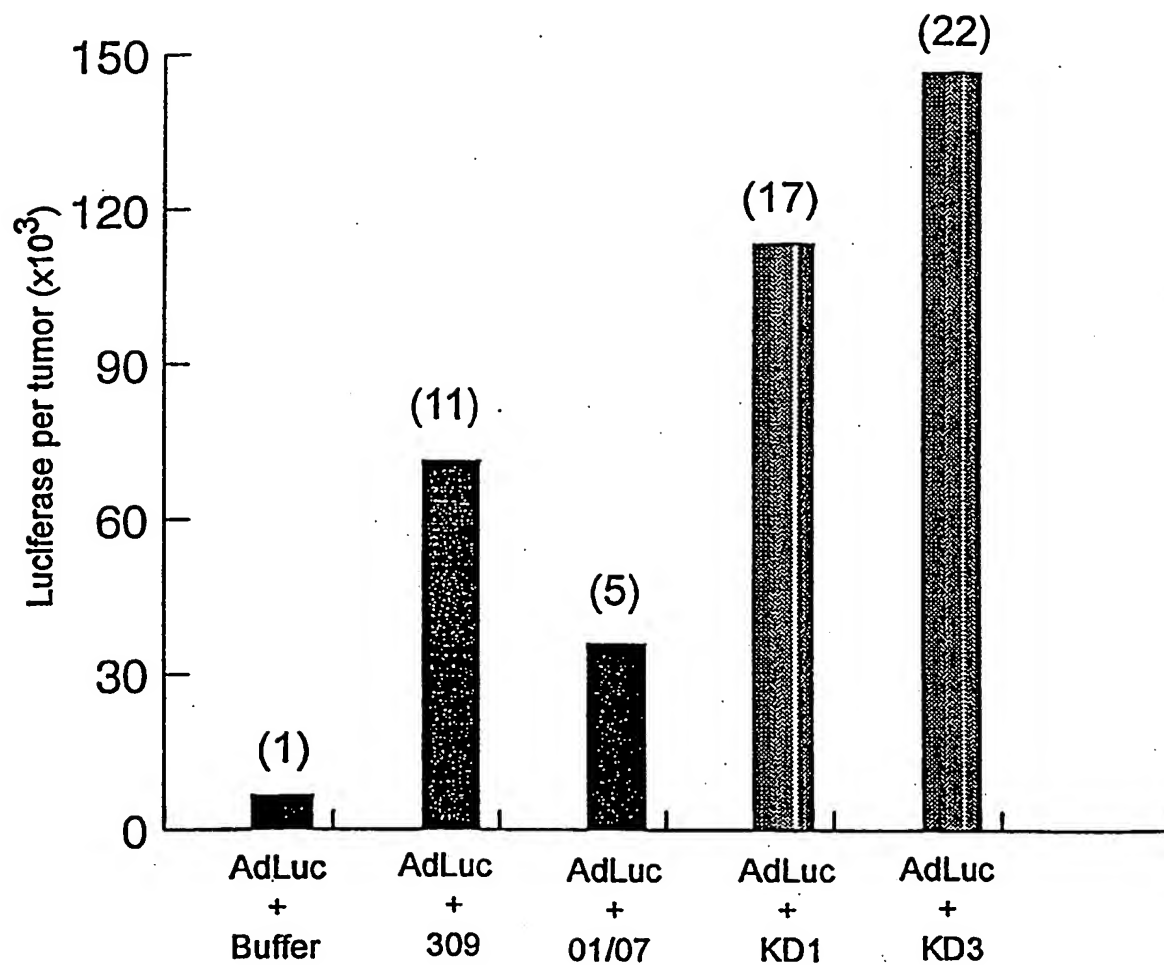


FIGURE 11

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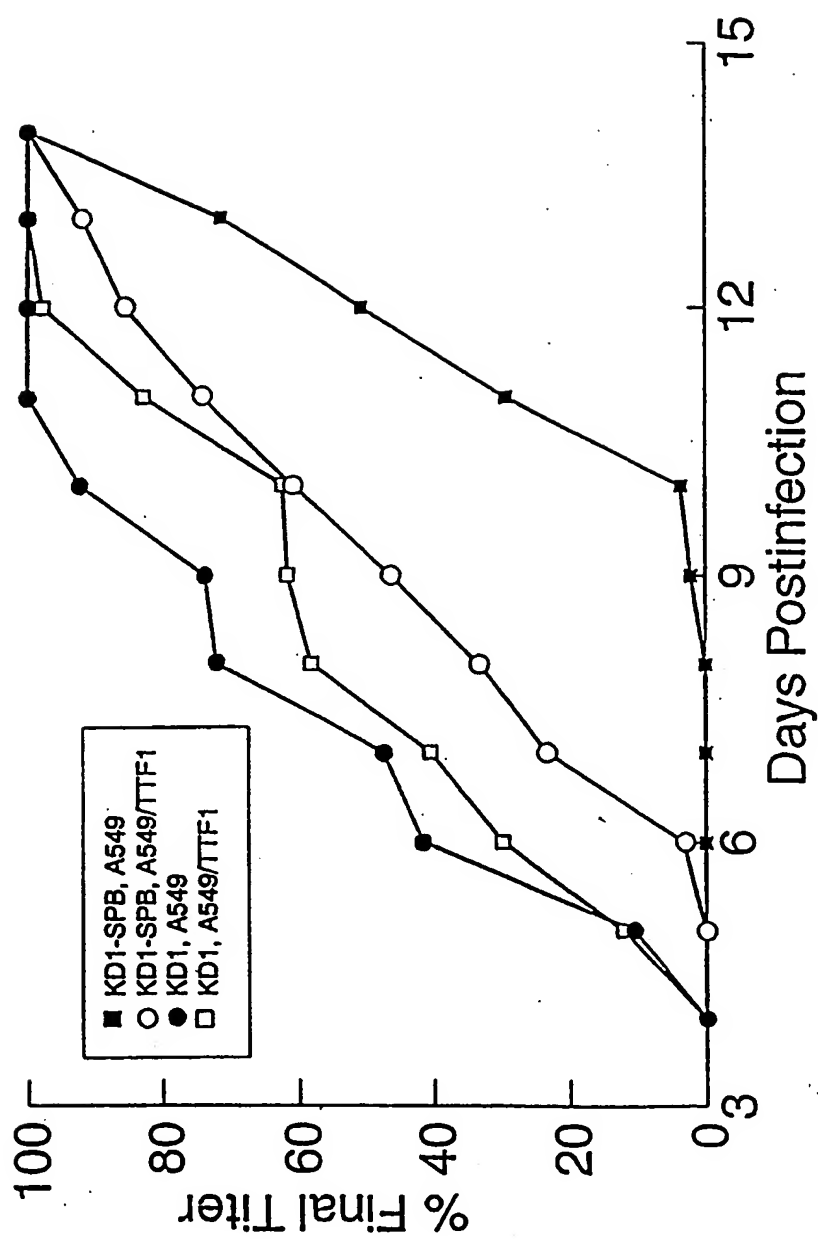
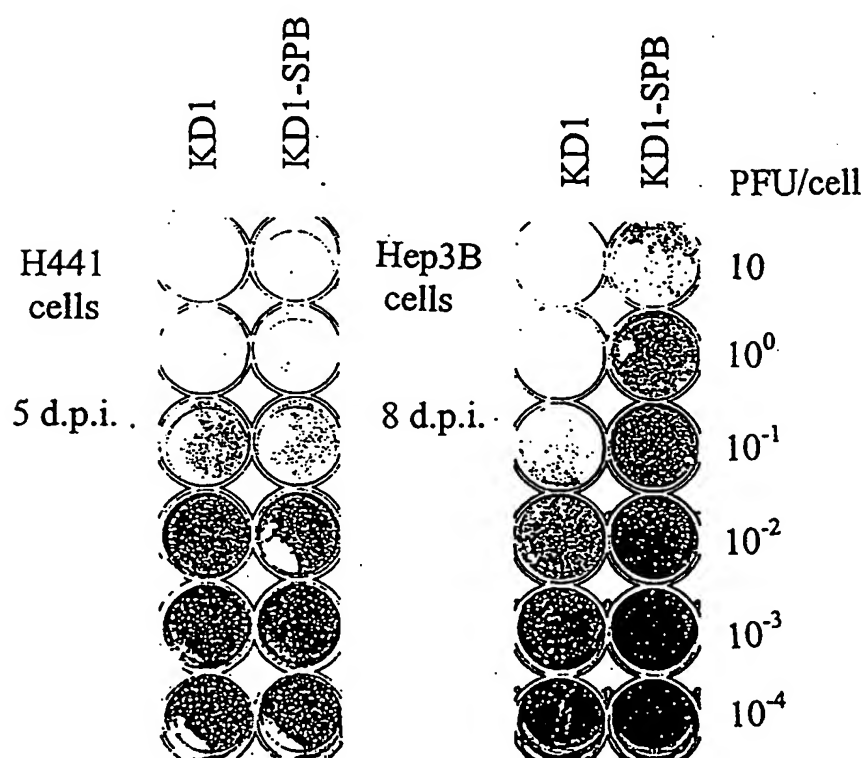


FIGURE 12



**KD1-SPB With the SPB Promoter in Place of the E4  
Promoter Grows on H44a Lung Cancer Cells with the  
TTF1 Transcription Factor**



**FIGURE 13**

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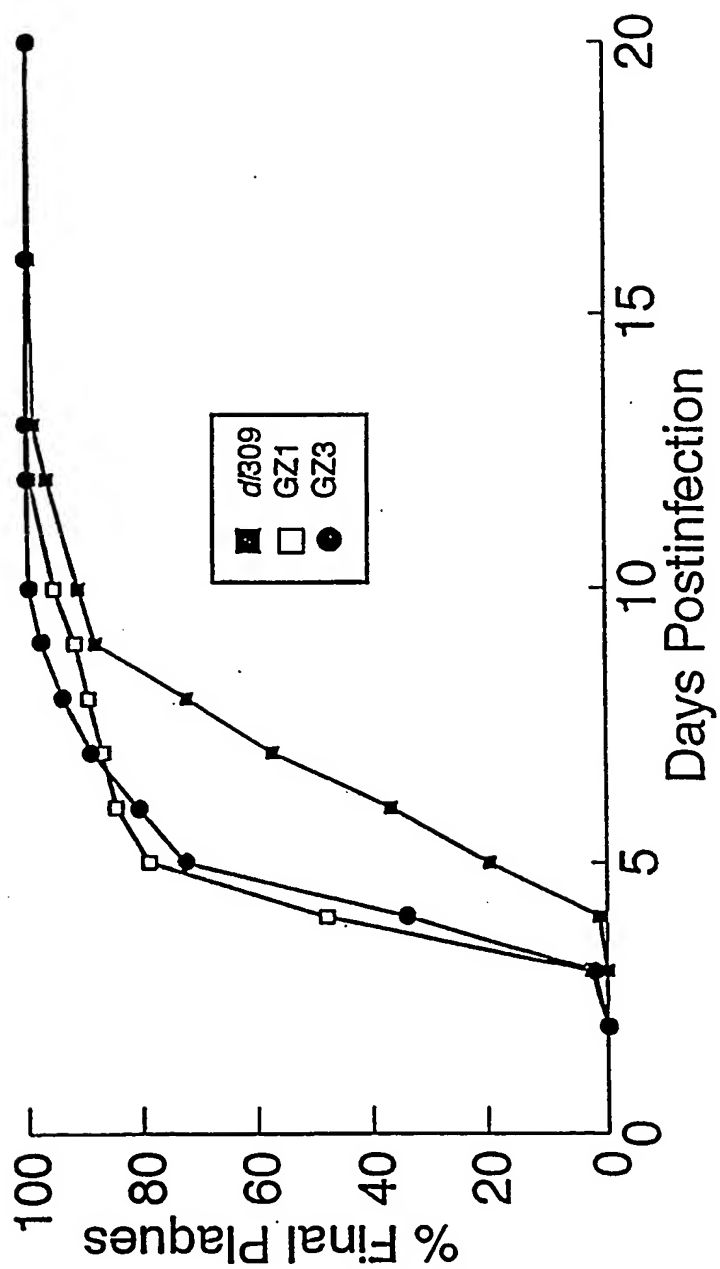


FIGURE 14

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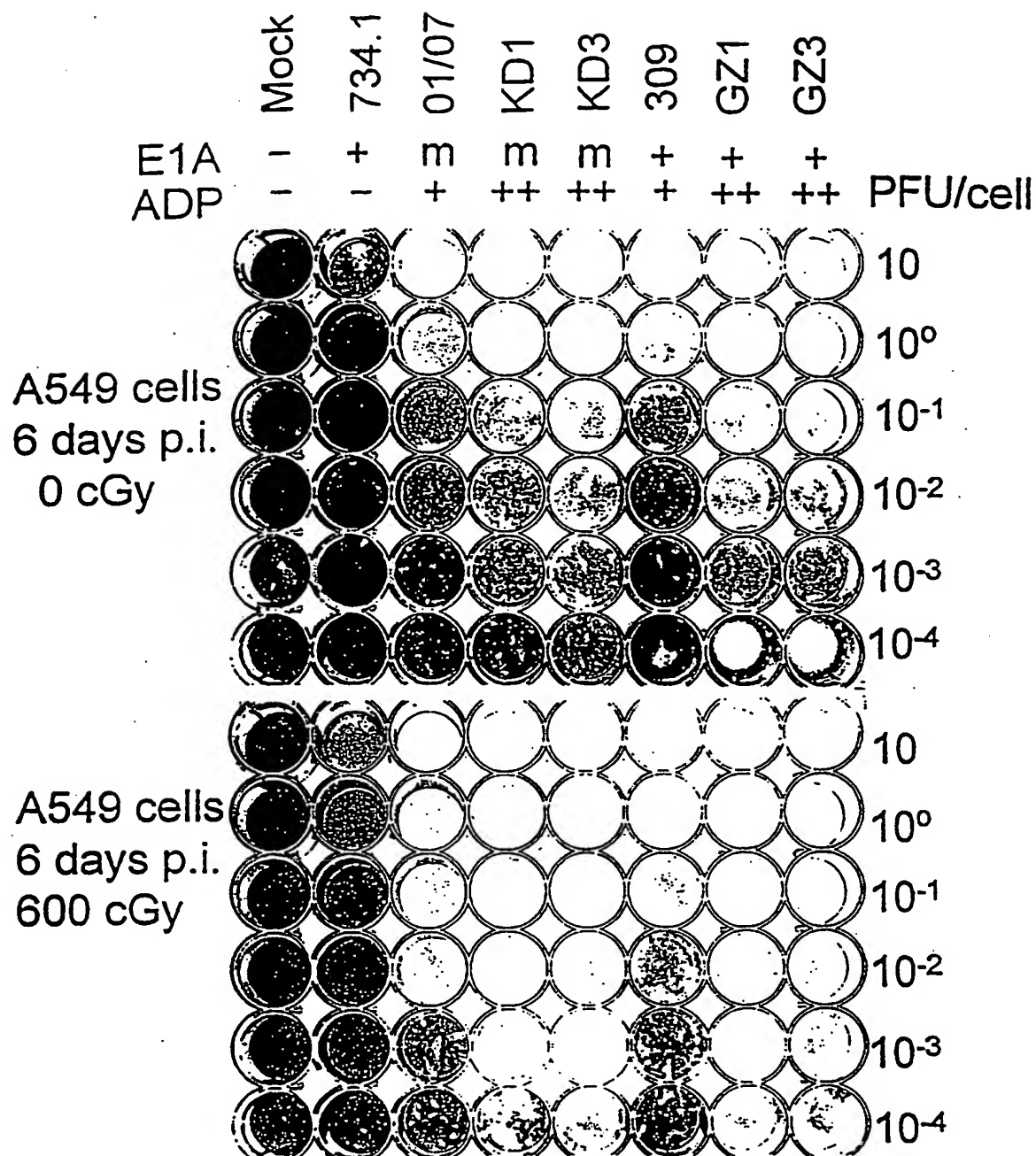


FIGURE 15

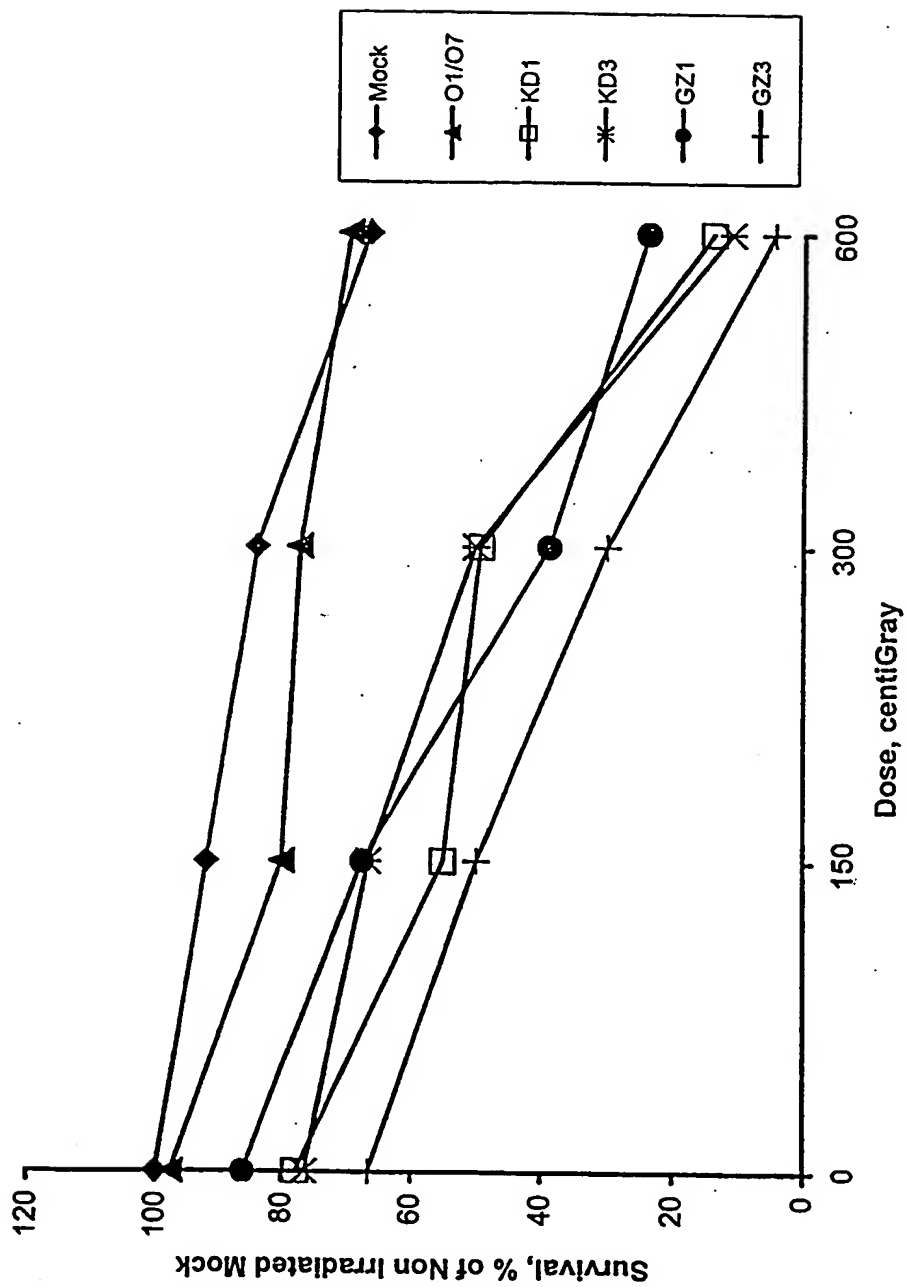


FIGURE 16

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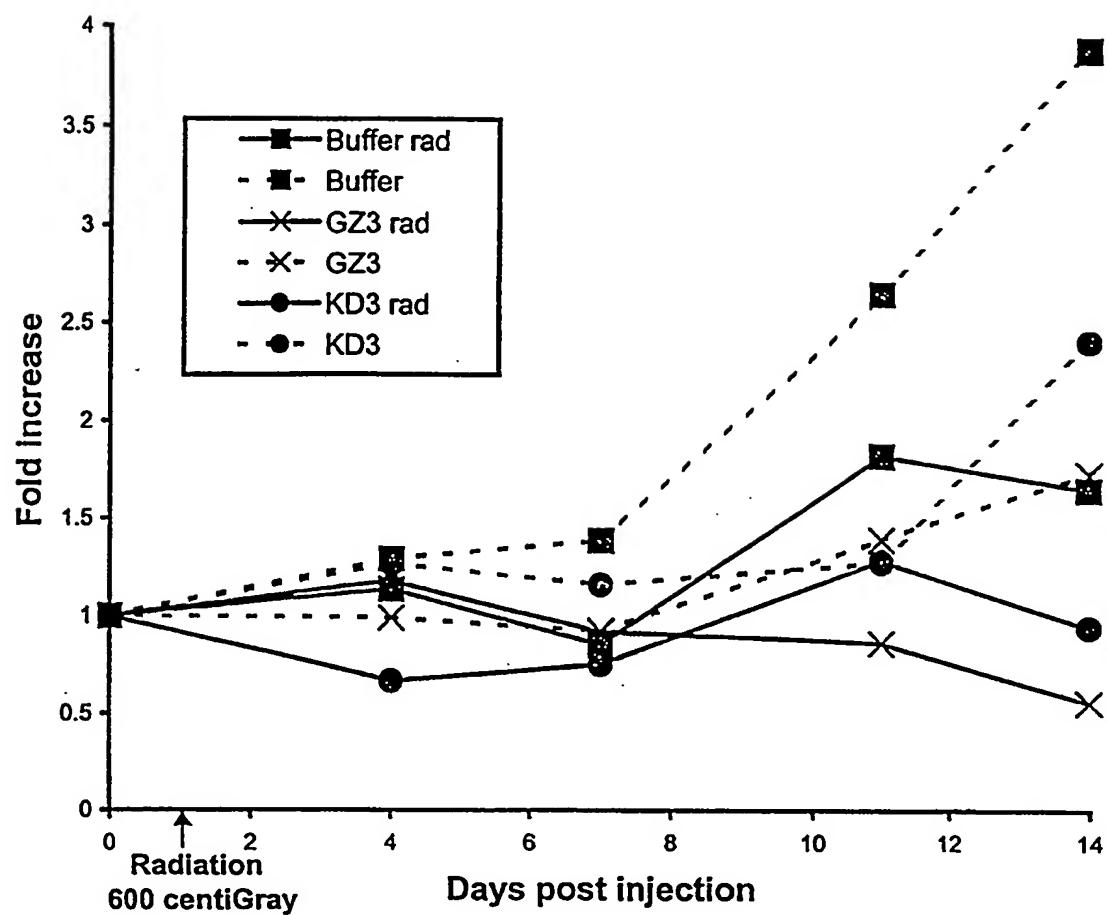


FIGURE 17

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## Ad2 Adenovirus Death Protein

### *Luminal Domain*

MTGSTIAPTTDYRNTTATGLTSALNLPQVHAFVND 35

*O - glycosylation*      *N - glycosylation*

WASLDMWWFSIALMFVCLIMWLICCLKRRRRARPP 70

*Transmembrane  
(Signal - Anchor)*

*Basic - Proline*

IYRPIIVLNPHNEKIHRLDGLKPCSLLLQYD 101

*Cytoplasmic - Nucleoplasmic Domain*

FIGURE 18A

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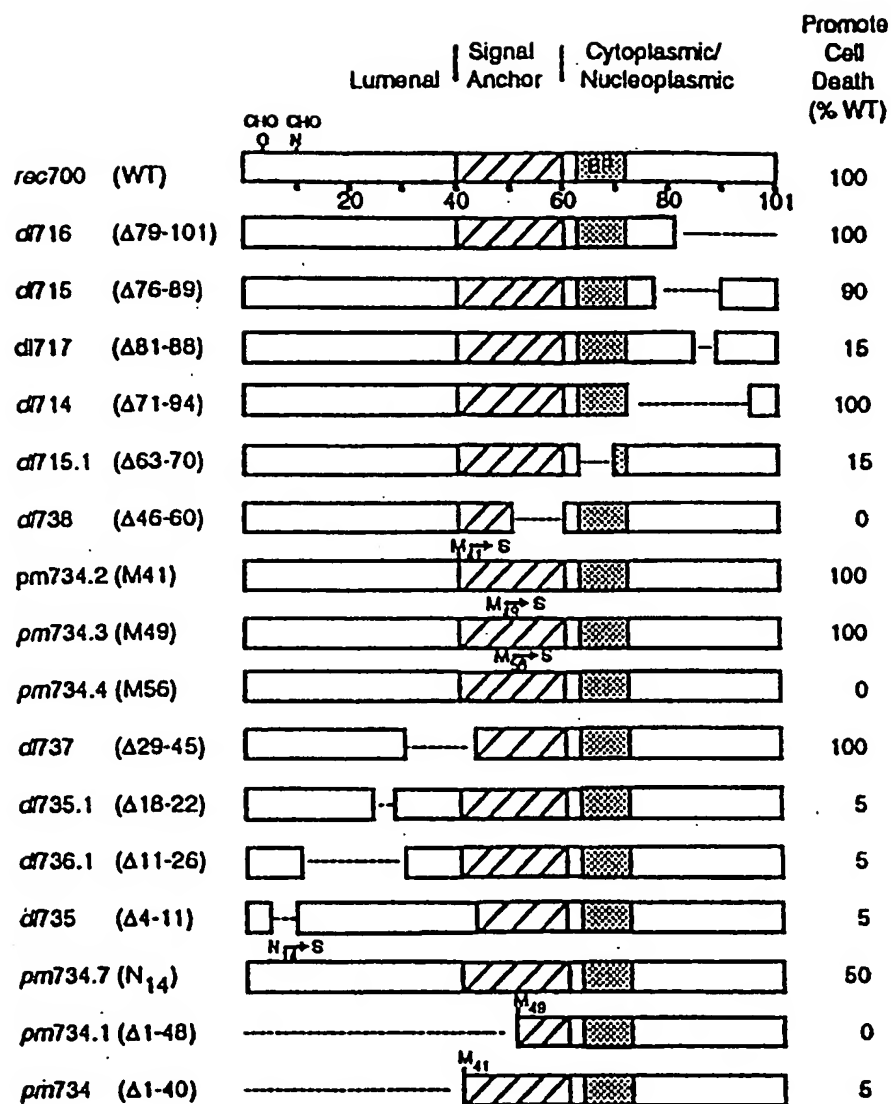


FIGURE 18B

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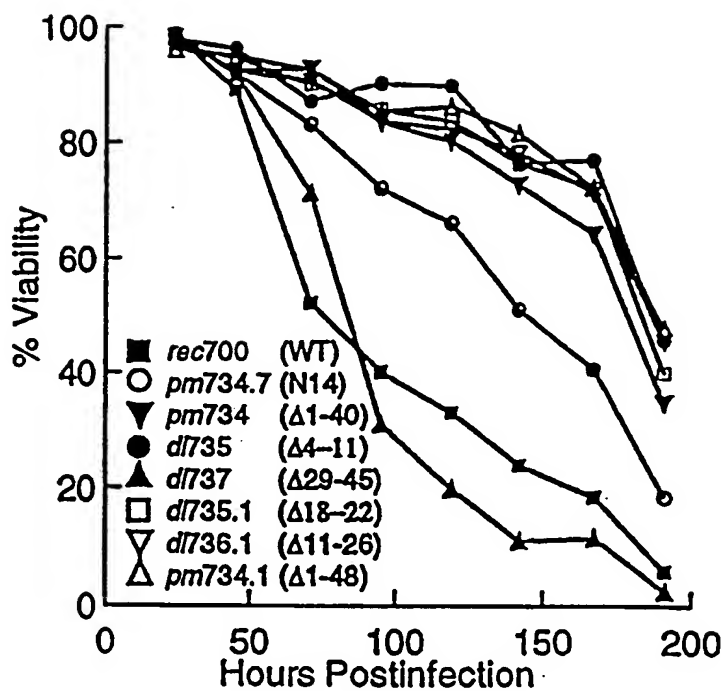


FIGURE 19A

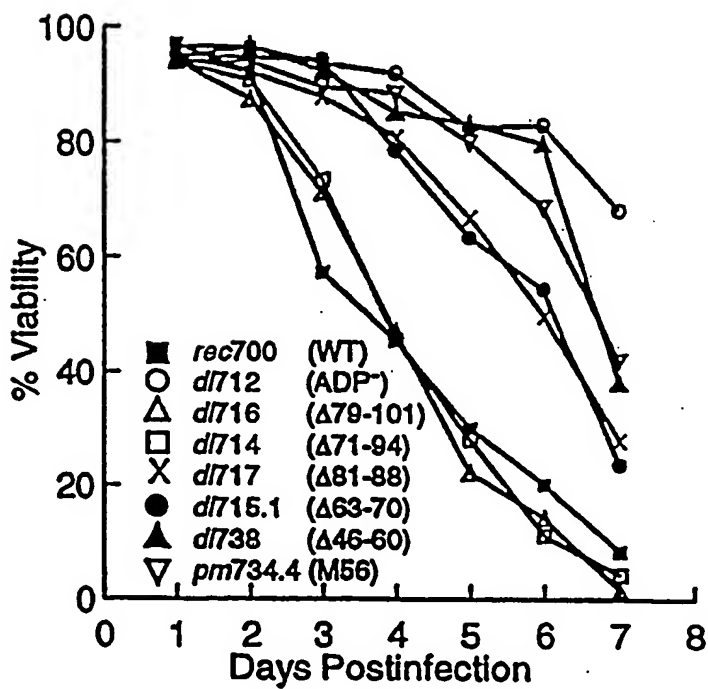


FIGURE 19B

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Seq ID No.

	10	20	30	40	50
5 Ad1	-----MVD	T VNSYNTATGL	TSALNLPQVS	TFVNNWANLG	MWWFSIALMF
6 Ad2	MTGSTIAPTT	DYRNTTATGL	TSALNLPQVH	AFVNDWASLD	MWWFSIALMF
7 Ad5	-----MTN	TTNAAAATGL	TSTINTPQVS	AFVNNWDNLG	MWWFSIALMF
8 Ad6	-----MVD	T VNSYNTATGL	KSALNLPQVH	AFVNDWASLG	MWWFSIALMF
9 dI716	MTGSTIAPTT	DYRNTTATGL	TSALNLPQVH	AFVNDWASLD	MWWFSIALMF
10 dI715	MTGSTIAPTT	DYRNTTATGL	TSALNLPQVH	AFVNDWASLD	MWWFSIALMF
11 dI714	MTGSTIAPTT	DYRNTTATGL	TSALNLPQVH	AFVNDWASLD	MWWFSIALMF
12 dI737	MTGSTIAPTT	DYRNTTATGL	TSALNLPQ--	-----	-----IALMF

	60	70	80	90	100
5 Ad1	VCLIIMWLSC	CLKRRRARPP	IYKPIIVLNP	NNDGIHRLDG	LNTCSFSFAV -
6 Ad2	VCLIIMWLIC	CLKRRRARPP	IYRPIIVLNP	HNEKIHRLDG	LKPCSLLLQY D
7 Ad5	VCLIIMWLIC	CLKRRRARPP	IYSPIIVLHP	NNDGIHRLDG	LKHMFFSLTV -
8 Ad6	VCLIIMWLIC	CLKRRRARPP	IYRPIIVLNP	HNEKIHRLDG	LKPCSLLLQY D
9 dI716	VCLIIMWLIC	CLKRRRARPP	IYRPIIVL--	-----	-----
10 dI715	VCLIIMWLIC	CLKRRRARPP	IYRPI-----	-----G	LKPCSLLLQY D
11 dI714	VCLIIMWLIC	CLKRRRARPP	-----	-----	-----SLLLQY D
12 dI737	VCLIIMWLIC	CLKRRRARPP	IYRPIIVLNP	HNEKIHRLDG	LKPCSLLLQY D

Seq. ID No.

17	aa 1-40 of Ad2 ADP	MTGSTIAPTT DYRNTTATGL TSALNLPQVH AFVNDWASLD
18	aa 41-59 of Ad2 ADP	MWWFSIALMF VCLIIMWLI
19	aa 63-70 of Ad2 ADP	KRRRARPP
20	aa 60-101 of Ad2 ADP	C CLKRRRARPP IYRPIIVLNP HNEKIHRLDG LKPCSLLLQY D

FIGURE 20

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LOCUS ad5 comple 35935 bp DNA SYN 06-FEB-1999  
DEFINITION ad5 complete genome  
ACCESSION ad5 comple  
KEYWORDS  
SOURCE Unknown.  
ORGANISM Unknown  
Unclassified.  
REFERENCE 1 (bases 1 to 35935)  
AUTHORS Self  
JOURNAL Unpublished.  
BASE COUNT 8367 a 10073 c 9761 g 7734 t  
ORIGIN

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1 CATCATCAAT AATATACCTT ATTTTGGATT GAAGCCAATA TGATAATGAG GGGGTGGAGT
61 TTGTGACGTG GCGCGGGGCG TGGGAACGGG GCGGGTGACG TAGTAGTGTG GCGGAAGTGT
121 GATGTTGCAA GTGTGGCGGA ACACATGTAA GCGACGGATG TGGCAAAAGT GACGTTTTTG
181 GTGTGCGCCG GTGTACACAG GAAGTGACAA TTTTCGCGCG GTTTTAGGCG GATGTTGTAG
241 TAAATTTGGG CGTAACCGAG TAAGATTTGG CCATTTTCGC GGGAAAACCTG AATAAGAGGA
301 AGTGAAATCT GAATAATTTT GTGTTACTCA TAGCGCGTAA TATTTGTCTA GGGCCGCGGG
361 GACTTTGACC GTTTACGTGG AGACTCGCCC AGGTGTTTTT CTCAGGTGTT TTCCGCGTTC
421 CGGGTCAAAG TTGGCGTTTT ATTATTATAG TCAGCTGACG TGTAGTGAT TATACCCGG
481 TGAGTTCTCT AAGAGGCCAC TCTTGAGTGC CAGCGAGTAG AGTTTTCTCC TCCGAGCCGC
541 TCCGACACCG GGACTGAAAA TGAGACATAT TATCTGCCAC GGAGGTGTTA TTACCGAAGA
601 AATGGCCGCC AGTCTTTTGG ACCAGCTGAT CGAAGAGGTA CTGGCTGATA ATCTTCCACC
661 TCCTAGCCAT TTTGAACCAC CTACCCTTCA CGAACTGTAT GATTTAGACG TGACGGCCCC
721 CGAAGATCCC AACGAGGAGG CGGTTTCGCA GATTTTTCCT GACTCTGTAA TGTGGCGGT
781 GCAGGAAGGG ATTGACTTAC TCACCTTTCC GCCCGCGCCC GGTTCCTCCG AGCCGCTCA
841 CCTTTCCCGG CAGCCCGAGC AGCCCGAGCA GAGAGCCTTG GGTCCGGTTT CTATGCCAAA
901 CCTTGTACCG GAGGTGATCG ATCTTACCTG CCACGAGGCT GGCTTTCAC CCAGTGACGA
961 CGAGGATGAA GAGGGTGAGG AGTTTGTGTT AGATTATGTG GAGCACCCCG GGCACGGTTG
1021 CAGGTCTTGT CATTATCACC GGAGGAATAC GGGGGACCCA GATATTATGT GTTCGCTTTG
1081 CTATATGAGG ACCGTGTGCA TGTTTGTCTA CAGTAAGTGA AAATTATGGG CAGTGGGTGA
1141 TAGAGTGGTG GGTTTGGTGT GGTAATTTTT TTTTAAATTT TTACAGTTTT GTGGTTTAAA
1201 GAATTTTGTA TTGTGATTTT TTTAAAAGGT CCTGTGCTCG AACCTGAGCC TGAGCCCGAG
1261 CCAGAACCGG AGCCTGCAAG ACCTACCCGC CGTCCTAAAA TGGCGCCTGC TATCCTGAGA
1321 CGCCCGACAT CACCTGTGTC TAGAGAATGC AATAGTAGTA CGGATAGCTG TGACTCCGGT
1381 CCTTCTAACA CACCTCCTGA GATACACCCG GTGGTCCCGC TGTGCCCAT TAAACCAATT
1441 GCCGTGAGAG TTGGTGGGCG TCGCCAGGCT GTGGAATGTA TCGAGGACTT GCTTAACGAG
1501 CCTGGGCAAC CTTTGGACTT GAGCTGTAAA CGCCCCAGGC CATAAGGTGT AAACCTGTGA
1561 TTGCGTGTGT GGTAAACGCC TTTGTTTGCT GAATGAGTTG ATGTAAGTTT AATAAAGGGT
1621 GAGATAATGT TTAACCTGCA TGGCGTGTTA AATGGGGCGG GGCTTAAAGG GTATATAATG
1681 CGCCGTGGGC TAATCTTGGT TACATCTGAC CTCATGGAGG CTTGGGAGTG TTTGGAAGAT
1741 TTTTCTGCTG TGCGTAACTT GCTGGAACAG AGCTCTAACA GTACCTCTTG GTTTTGGAGG
1801 TTTCTGTGGG GCTCATCCCA GGCAAAGTTA GTCTGCAGAA TTAAGGAGGA TTACAAGTGG
1861 GAATTTGAAG AGCTTTTGAA ATCCTGTGTT GAGCTGTTTG ATTCTTTGAA TCTGGGTCAC
1921 CAGGCGCTTT TCCAAGAGAA GGTCATCAAG ACTTTGGATT TTTCCACACC GGGGCGCGCT
1981 GCGGCTGCTG TTGCTTTTTT GAGTTTTATA AAGGATAAAT GGAGCGAAGA AACCCATCTG
2041 AGCGGGGGGT ACCTGCTGGA TTTTCTGGCC ATGCATCTGT GGAGAGCGGT TGTGAGACAC
2101 AAGAAATCGC TGCTACTGTT GTCTTCCGTC CGCCCGGCGA TAATACCGAC GGAGGAGCAG
2161 CAGCAGCAGC AGGAGGAAGC CAGGCGGCGG CGGCAGGAGC AGAGCCCATG GAACCCGAGA
2221 GCCGCGCTGG ACCCTCGGGA ATGAATGTTG TACAGGTGGC TGAAGTGTAT CCAGAAGTGA
2281 GACGCAATTT GACAATTACA GAGGATGGGC AGGGGCTAAA GGGGGTAAAG AGGGAGCGGG
2341 GGGCTTGTGA GGCTACAGAG GAGGCTAGGA ATCTAGCTTT TAGCTTAATG ACCAGACACC
2401 GTCCTGAGTG TATTACTTTT CAACAGATCA AGGATAATTG CGCTAATGAG CTTGATCTGC
2461 TGGCGCAGAA GTATTCCATA GAGCAGCTGA CCACCTACTG GCTGCAGCCA GGGGATGATT
2521 TTGAGGAGGC TATTAGGGTA TATGCAAAGG TGGCACTTAG GCCAGATTGC AAGTACAAGA
2581 TCAGCAAACT TGTAAATATC AGGAATGTGT GCTACATTTT TGGGAACGGG CCGGAGGTGG
2641 AGATAGATAC GGAGGATAGG GTGGCCTTTA GATGTAGCAT GATAAATATG TGGCCGGGGG
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2761 GTACGGTTTT CCTGGCCAAT ACCAACCTTA TCCTACACGG TGTAAGCTTC TATGGGTTTA
2821 ACAATACCTG TGTGGAAGCC TGGACCGATG TAAGGGTTTC GGGCTGTGCC TTTTACTGCT
2881 GCTGGAAGGG GGTGGTGTGT CGCCCCAAAA GCAGGGCTTC AATTAAGAAA TGCCTCTTGT
2941 AAAGGTGTAC CTTGGGTATC CTGTCTGAGG GTAACCTCCAG GGTGCGCCAC AATGTGGCCT
3001 CCGACTGTGG TTGCTTCATG CTAGTGA AAA GCGTGGCTGT GATTAAGCAT AACATGGTAT
3061 GTGGCAACTG CGAGGACAGG GCCTCTCAGA TGCTGACCTG CTCGACGGC AACTGTCACC
3121 TGCTGAAGAC CATTACGTA GCCAGCCACT CTCGCAAGGC CTGGCCAGTG TTTGAGCATA
3181 ACATACTGAC CCGCTGTTCC TTGCATTTGG GTAACAGGAG GGGGGTGTTC CTACCTTACC
3241 AATGCAATTT GAGTCACACT AAGATATTGC TTGAGCCCGA GAGCATGTCC AAGGTGAACC
3301 TGAACGGGGT GTTTGACATG ACCATGAAGA TCTGGAAGGT GCTGAGGTAC GATGAGACCC
3361 GCACCAGGTG CAGACCCTGC GAGTGTGGCG GTAAACATAT TAGGAACCAG CCTGTGATGC
3421 TGGATGTGAC CGAGGAGCTG AGGCCCGATC ACTTGGTGTG GGCCTGCACC CGCGCTGAGT
3481 TTGGCTCTAG CGATGAAGAT ACAGATTGAG GTACTGAAAT GTGTGGCGT GGCTTAAGGG
3541 TGGGAAGAA TATATAAGGT GGGGGTCTTA TGTAGTTTTG TATCTGTTTT GCAGCAGCCG
3601 CCGCCGCCAT GAGCACC AAC TCGTTTGATG GAAGCATTGT GAGCTCATAT TTGACAACGC
3661 GCATGCCCCC ATGGGCCGGG GTGCGTCAGA ATGTGATGGG CTCCAGCATT GATGGTCCGC
3721 CCGTCCCTGC CGCAAACCTCT ACTACCTTGA CCTACGAGAC CGTGTCTGGA ACGCGTTGG
3781 AGACTGACGC CTCGCGCCGC GCTTCAGCCG CTGCAGCCAC CGCCCGCGG ATTGTGACTG
3841 ACTTTGCTTT CCTGAGCCCG CTTCGCAAGCA GTGCAGCTTC CCGTTCATCC GCCCGCGATG
3901 ACAAGTTGAC GGCTCTTTTG GCACAATTGG ATTCTTTGAC CCGGGAACCT AATGTGTTTT
3961 CTCAGCAGCT GTTGGATCTG CGCCAGCAGG TTTCTGCCCT GAAGGCTTCC TCCCCTCCCA
4021 ATGCGGTTTA AAACATAAAT AAAAAACCAG ACTCTGTTTG GATTTGATC AAGCAAGTGT
4081 CTTGCTGTCT TTATTTAGGG GTTTTGCGCG CGCGTAGGC CCGGACCAG CGGTCTCGGT
4141 CGTTGAGGGT CCTGTGTATT TTTTCCAGGA CGTGGTAAAG GTGACTCTGG ATGTTTCAGT
4201 ACATGGGCAT AAGCCCGTCT CTGGGGTGA GGTAGCACC CTGCAGAGCT TCATGCTGCG
4261 GGGTGGTGT GTAGATGATC CAGTCGTAGC AGGAGCGCTG GCGGTGGTGC CTAAAAATGT
4321 CTTTCAGTAG CAAGCTGATT GCCAGGGGCA GGCCCTTGGT GTAAGTGTTC ACAAAGCGGT
4381 TAAGCTGGGA TGGGTGCATA CGTGGGGATA TGAGATGCAT CTTGGACTGT ATTTTLAGGT
4441 TGGCTATGTT CCCAGCCATA TCCCTCCGGG GATTTCATGT GTGCAGAAC ACCAGCACAG
4501 TGTATCCGGT GCACTTGGA AATTGTGTC GTAGCTTAGA AGGAAATGCG TGAAGAATC
4561 TGGAGACGCC CTTGTGACCT CCAAGATTTT CCATGCATTC GTCCATAATG ATGGCAATGG
4621 GCCCACGGGG GCGCGCTCGG GCGAAGATAT TTCTGGGATC ACTAACGTCA TAGTTGTGTT
4681 CCAGGATGAG ATCGTCATAG GCCATTTTTA CAAAGCGCGG GCGGAGGGTG CCAGACTGCG
4741 GTATAATGGT TCCATCCGGC CCAGGGGCGT AGTTACCTC ACAGATTTGC ATTTCCACG
4801 CTTTGAGTTC AGATGGGGG ATCATGTCTA CCTGCGGGG GATGAAGAAA ACGGTTTCCG
4861 GGGTAGGGGA GATCAGCTGG GAAGAAAGCA GGTTCCTGAG CAGCTGCGAC TTACCGCAGC
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5041 CCTGACCAA ATCCGCCAGA AGGCGCTCGC CGCCAGCGA TAGCAGTTCT TGCAAGGAAG
5101 CAAAGTTTTT CAACGGTTTG AGACCGTCCG CCGTAGGCAT GCTTTTGAGC GTTTGACCAA
5161 GCAGTTCCAG GCGGTCCAC AGCTCGGTCA CCTGCTCTAC GGCATCTCGA TCCAGCATAT
5221 CTCCTCGTTT CGCGGGTTGG GCGGGCTTTC GCTGTACGGC AGTAGTCGGT GCTCGTCCAG
5281 ACGGGCCAGG GTCATGTCTT TCCACGGGCG CAGGGTCCTC GTCAGCGTAG TCTGGGTAC
5341 GGTGAAGGGG TGCCTCCGG GCTGCGCGCT GGCCAGGGTG CGCTTGAGGC TGGTCTGCT
5401 GGTGCTGAAG CGCTGCCGGT CTTGCGCCCTG CGCGTCCGGC AGGTAGCATT TGACCATGGT
5461 GTCATAGTCC AGCCCTCCG CGGCGTGCC CTTGGCGCGC AGCTTGCCCT TGGAGGAGGC
5521 GCGCACGAG GGGCAGTGCA GACTTTTGAG GCGTAGAGC TTGGGCGCGA GAAATACCGA
5581 TTCGGGGGAG TAGGCATCCG CGCCGAGGC CCGCAGACG GTCTCGCATT CCACGAGCCA
5641 GGTGAGCTCT GGCCGTTCCG GGTCAAAAAC CAGGTTTCCC CCATGCTTTT TGATGCGTTT
5701 CTTACCTCTG GTTTCCATGA GCGGGTGTCC ACGCTCGGTG ACGAAAAGGC TGTCCGTGTC
5761 CCGGTATACA GACTTGAGAG GCCTGTCTC GAGCGGTGTT CCGCGGTCTT CCTCGTATAG
5821 AAACCTGGAC CACTCTGAGA CAAAGGCTCG CGTCCAGGCC AGCACGAAGG AGGCTAAGTG
5881 GGAGGGGTAG CGGTCTGTTT CCACTAGGGG GTCCACTCGC TCCAGGGTGT GAAGACACAT
5941 GTCGCCCTCT TCGGCATCAA GGAAGGTGAT TGGTTTGTAG GTGTAGGCCA CGTGACCGGG
6001 TGTTCCTGAA GGGGGGCTAT AAAAGGGGT GGGGGCGCGT TCGTCTCAC TCTCTCCGC
6061 ATCGCTGTCT GCGAGGGCCA GCTGTTGGGG TGAGTACTCC CTCTGAAAAG CGGGCATGAC

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6121 TTCTGCGCTA AGATTGTCAG TTTCCAAAAA CGAGGAGGAT TTGATATTCA CCTGGCCCGC  
6181 GGTGATGCCT TTGAGGGTGG CCGCATCCAT CTGGTCAGAA AAGACAATCT TTTTGTGTGTC  
6241 AAGCTTGGTG GCAAAACGACC CGTAGAGGGC GTTGGACAGC AACTTGGCGA TGGAGCGCAG  
6301 GGTTTGGTTT TTGTGCGGAT CGGCGCGCTC CTTGGCCGCG ATGTTTAGCT GCACGTATTC  
6361 GCGCGCAACG CACCGCCATT CGGGAAGAC GGTGGTGGC TCGTCGGGCA CCAGGTGCAC  
6421 GCGCCAACCG CGGTGTGTGA GGTGACAAAG GTCAACGCTG GTGGCTACCT CTCGCGTAG  
6481 GCGCTCGTTG GTCCAGCAGA GCGCGCCGCC CTTGCGCGAG CAGAATGGCG GTAGGGGGTC  
6541 TAGCTGCGTC TCGTCCGGGG GGTCTGCGTC CACGGTAAAG ACCCGGGCA GCAGGCGCGC  
6601 GTCGAAGTAG TCTATCTTGC ATCCTTGCAA GTCTAGCGCC TGCTGCCATG CGCGGGCGGC  
6661 AAGCGCGCGC TCGTATGGGT TGAGTGGGGG ACCCATGGC ATGGGGTGG TGAGCGCGGA  
6721 GCGGTACATG CCGCAAATGT CGTAAACGTA GAGGGGCTCT CTGAGTATTC CAAGATATGT  
6781 AGGGTAGCAT CTTCCACCGC GGATGCTGGC GCGCACGTAA TCGTATAGTT CGTCCGAGGG  
6841 AGCGAGGAGG TCGGACCGA GGTGCTACG GCGGGGCTGC TCTGCTCGGA AGACTATCTG  
6901 CCTGAAGATG GCATGTGAGT TGGATGATAT GGTGGACGC TGAAGACGT TGAAGCTGGC  
6961 GTCTGTGAGA CCTACCGGT CACGCACGAA GGAGGCGTAG GAGTCGCGCA GCTTGTGTAC  
7021 CAGCTCGGCG GTGACCTGCA CGTCTAGGCG GCAGTAGTCC AGGGTTTCCT TGATGATGTC  
7081 ATACTTATCC TGTCCTTTT TTTTCCACAG CTCGCGGTTG AGGACAACT CTTCCGGGTC  
7141 TTTCCAGTAC TCTTGGATCG GAAACCCGTC GGCCTCCGAA CGGTAAGAGC CTAGCATGTA  
7201 GAACGTGGTG ACGGCTGGT AGGCGCAGCA TCCCTTTTCT ACGGGTAGCG CGTATGCGCTG  
7261 CGCGGCTTTC CGGAGCGAGG TGTGGGTGAG CGCAAAGGTG TCCCTGACCA TGACTTTGAG  
7321 GTACTGGTAT TTGAAGTCAG TGTCGTGCGA TCCGCCCTGC TCCAGAGCA AAAAGTCCGT  
7381 GCGCTTTTGT GAACGCGGAT TTGGCAGGGC GAAGGTGACA TCGTTGAAGA GTATCTTTCC  
7441 CGCGCGAGGC ATAAAGTTGC GTGTGATGCG GAAGGGTCCC GGCACCTCG AACGGTGTGT  
7501 AATTACCTGG GCGGCGAGCA CGATCTCGTC AAAGCCGTTG ATGTTGTGGC CCACAATGTA  
7561 AAGTTCCAAG AAGCGCGGGA TGCCCTTGAT GGAAGGCAAT TTTTAAAGTT CCTCGTAGGT  
7621 GAGTCTTCA GGGGAGCTGA GCCCGTGCTC TGAAAGGGCC CAGTCTGCAA GATGAGGGTT  
7681 GGAAGCGACG AATGAGCTCC ACAGGTCACG GGCCATTAGC ATTTGCAGGT GGTCGCGAAA  
7741 GGTCTTAAC TGCGGACCTA TGCCCATTTT TTCTGGGGTG ATGCAGTAGA AGGTAAAGCGG  
7801 GTCTTGTTC CAGCGGTCCC ATCCAAGGT CGCGGCTAGG TCTCGCGCGG CAGTCACTAG  
7861 AGGCTCATCT CCGCGAAGT TCATGACCAG CATGAAGGCG ACAGAGTGT TCCCAAGGC  
7921 CCCCATCCA GTATAGGTCT CTACATCGTA GGTGACAAAG AGACGCTCGG TGCGAGGATG  
7981 CGAGCCGATC GGAAGAAGT GGATCTCCCG CCACCAATTG GAGGAGTGGC TATTGATGTG  
8041 GTGAAGTAG AAGTCCCTGC GACGGGCCGA ACACCTCGTC TGGCTTTTGT AAAACGTCG  
8101 GCAGTACTGG CAGCGGTGCA CGGGCTGTAC ATCCTGCACG AGGTTGACCT GACGACCGCG  
8161 CACAAGGAAG CAGAGTGGGA ATTGAGCCC CTCGCTGGC GGGTTTGGCT GGTGGTCTTC  
8221 TACTTCGGCT GCTTGTCTT GACCGTCTGG CTGCTCGAGG GGAGTTACGG TGGATCGGAC  
8281 CACCAACCGG CCGGAGCCCA AAGTCCAGT GTCCGCGCGC GCGGGTCGGA GCTTGTGAC  
8341 AACATCGCGC AGATGGGAGC TGTCATGGT CTGGAGCTCC CCGGCGTCA GGTGAGCGG  
8401 GAGCTCTGCG AGGTTTACCT CGCATAGACG GGTCAGGGCG CCGGCTAGAT CCAGGTGATA  
8461 CCTAATTTC AGGGGCTGGT TGGTGGCGGC GTCGATGGCT TGCAAGAGGC CGCATCCCCG  
8521 CGGCGCGACT ACGGTACCGC GCGGCGGGCG GTGGGCGCG GGGGTGTCCT TGGATGATGC  
8581 ATCTAAAGC GGTGACGCG GCGAGCCCC GGAGGTAGGG GGGGCTCCGG ACCCGCCGGG  
8641 AGAGGGGGCA GGGGACGTC GCGGCGCGC GCGGGCAGGA GCTGGTGTG CCGCGGTAGG  
8701 TTGCTGGCGA ACGCGACGAC GCGGCGGTTG ATCTCTGAA TCTGGCGCCT CTGCGTGAAG  
8761 ACGACGGGCC CGGTGAGCTT GAGCCTGAAA GAGAGTTGGA CAGAATCAAT TTCGGTGTG  
8821 TTGACGGCGG CCTGGCGCAA AATCTCTGCG ACGTCTCTG AGTTGTCTG ATAGGCGATC  
8881 TCGGCCATGA ACTGCTCGAT CTCTTCTCC TGGAGATCTC CGCGTCCGGC TCGTCCACG  
8941 GTGGCGGCGA GGTGTTGGA AATGCGGGCC ATGAGCTGCG AGAAGGCGTT GAGGCTCCG  
9001 TCGTTCCAGA CGCGGCTGTA GACCAAGCCC CTTTCGGCAT CGCGGGCGCG CATGACCACC  
9061 TGCGCGAGAT TGAGCTCCAC GTGCGGGCG AAGACGGCGT AGTTTCGCAG GCGCTGAAAG  
9121 AGGTAGTTGA GGGTGGTGGC GGTGTGTTCT GCCACGAAGA AGTACATAAC CCAGCGTCG  
9181 AACGTGGATT CGTTGATATC CCCCAAGGCC TCAAGGCGCT CCATGGCCTC GTAGAAGTCC  
9241 ACGGCGAAGT TGA AAAACTG GGAGTTGCGC GCGGACACGG TTAACCTCCT CTCCAGAAGA  
9301 CGGATGAGCT CCGCGACAGT GTCGCGCACC TCGCGCTCAA AGGCTACAGG GGCCTCTTCT  
9361 TCTTCTTCAA TCTCTCTTC CATAAGGGCC TCCCTTCTT CTCTTCTG CCGCGGTGGG  
9421 GGAGGGGGGA CACGCGGCG ACGACGGCG ACCGGGAGGC GGTGACAAA GCGCTCGATC  
9481 ATCTCCCCGC GCGGACGGCG CATGGTCTCG GTGACGGCG GCGCGTTCTC GCGGGGGCGC

FIGURE 21  
(SHEET 3)

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9541 AGTTGGAAGA CGCCGCCCGT CATGTCCCGG TTATGGGTTG GCGGGGGGCT GCCATGCGGC  
 9601 AGGGATACGG CGCTAACGAT GCATCTCAAC AATTGTTGTG TAGGTACTCC GCCGCCGAGG  
 9661 GACCTGAGCG AGTCCGCATC GACCGGATCG GAAAACCTCT CGAGAAAGGC GTCTAACCAAG  
 9721 TCACAGTCGC AAGGTAGGCT GAGCACCCTG GCGGGCGGCA GCGGGCGGCG GTCGGGGTTG  
 9781 TTTCTGGCGG AGGTGCTGCT GATGATGTAA TTAAGTAGG CGGTCTTGAG ACGGCGGATG  
 9841 GTCGACAGAA GCACCATGTC CTTGGGTCCG GCCTGCTGAA TCGCGAGGCG GTCGGCCATG  
 9901 CCCCAGGCTT CGTTTTGACA TCGGCGCAGG TCTTTGTAGT AGTCTTGAT GAGCCTTTCT  
 9961 ACCGGCACTT CTTCTCTCC TTCTCTTGT CTTGCATCTC TTGCATCTAT CGCTGCGGCG  
 10021 GCGGCGGAGT TTGGCCGTAG GTGGCGCCCT CTTCCTCCCA TCGGTGTGAC CCCGAAGCCC  
 10081 CTCATCGGCT GAAGCAGGCG TAGGTGCGCG ACAACGCGCT CGGCTAATAT GGCCTGCTGC  
 10141 ACCTGCGTGA GGGTAGACTG GAAGTCATCC ATGTCCACAA AGCGGTGGTA TCGGCCCGTG  
 10201 TTGATGGTGT AAGTGCAGTT GGCCATAACG GACCAGTTAA CGGTCTGCTG ACCCGGCTGC  
 10261 GAGAGCTCGG TGTACCTGAG ACGCGAGTAA GCCCTCGAGT CAAATACGTA GTCGTTGCAA  
 10321 GTCCGCACCA GGTACTGGTA TCCACCAAAA AAGTGCAGCG GCGGCTGGCG GTAGAGGGGC  
 10381 CAGCGTAGGG TGGCCGGGCG TCCGGGGGCG AGATCTTCCA ACATAAGGCG ATGATATCCG  
 10441 TAGATGTACC TGGACATCCA GGTGATGCCG GCGGCGGTGG TGGAGGCGCG CGGAAAGTCG  
 10501 CGGACGCGGT TCCAGATGTT GCGCAGCGGC AAAAAGTGCT CCATGGTCGG GACGCTCTGG  
 10561 CCGGTCAGGC GCGCGCAATC GTTGACGCTC TAGACCGTGC AAAAGGAGAG CTTGTAAGCG  
 10621 GGCACCTCTC CGTGGTCTGG TGGATAAATT CGCAAGGGTA TCATGGCGGA CGACCGGGGT  
 10681 TCGAGCCCGG TATCCGGGCG TCCGCCGTGA TCCATGCGGT TACCGCCGCG GTGTGGAACC  
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 10921 GCGGGACCCC CGGTTCGAGT CTCGGACCGG CCGGACTGCG GCGAACGGGG GTTTGCTTCC  
 10981 CCGTCATGCA AGACCCCGCT TGCAAAATCC TCCGGAACA GGGACGAGCC CCTTTTTTGC  
 11041 TTTTCCAGAG TGCATCCGCT GCTGCGCAGC ATGCGCCCC CTCCTCAGCA GCGGCAAGAG  
 11101 CAAGAGCAGC GGCAGACATG CAGGGCACCC TCCCTCTCTC CTACCGGCTC AGGAGGGGCG  
 11161 ACATCCGCGG TTGACGCGGC AGCAGATGGT GATTACGAAC CCCCAGCGCG CCGGGCCCGG  
 11221 CACTACCTGG ACTTGAGGGA GGGCGAGGCG CTGGCGCGGC TAGGAGCGCC CTCTCTGAG  
 11281 CGGTACCCAA GGGTGCAGCT GAAGCGTGAT ACGCGTGAGG CGTACGTGCC GCGGCAGAAC  
 11341 CTGTTTTCGG ACCGCGAGGG AGAGGAGCCC GAGGAGATGC GGGATCGAAA GTTCCACGCA  
 11401 GGGCGCGAGC TGCAGCATGG CCTGAATCCG GAGCGGTTGC TCGCGAGGGA GGACTTTGAG  
 11461 CCGGACGCGC GAACCGGGAT TAGTCCCGCG CGCGCACACG TGGCGGCCCG GACCTGGTA  
 11521 ACCGCATACG AGCAGACGGT GAACAGGAG ATTAACCTTC AAAAAAGCTT TAACAACCAC  
 11581 GTGCGTACGC TTGTGGCGCG CGAGGAGGTG GCTATAGGAC TGATGCATCT GTGGGACTTT  
 11641 GTAAGCGCGC TGGAGCAAAA CCCAAATAGC AAGCCGCTCA TGGCGCAGCT GTTCTTTATA  
 11701 GTGCAGCACA GCAGGGACAA CGAGGCATTC AGGGATGCGC TGCTAAACAT AGTAGAGCCC  
 11761 GAGGGCCGCT GGCTGCTCGA TTTGATAAAC ATCCTGCAGA GCATAGTGGT GCAGGAGCGC  
 11821 AGCTTTGAGC TGGCTGACAA GGTGGCCGCG ATCAACTATT CCATGCTTAG CCTGGGCAAG  
 11881 TTTTACGCCC GCAAGATATA CCATACCCCT TACGTTCCCA TAGACAAGGA GGTAAGATC  
 11941 GAGGGGTTCT ACATGCGCAT GCGCTGGAAG GTGCTTACCT TGAGCGACGA CCTGGGCGTT  
 12001 TATCGCAACG AGCGCATCCA CAAGGCGGTG AGCGTGAGCC GGCGGCGCGA GCTCAGCGAC  
 12061 CGCGAGCTGA TGCACAGCCT GCAAAGGGCC CTGGCTGGCA CCGGCAGCGG CGATAGAGAG  
 12121 GCGGAGTCTT ACTTTGACGC GGGCGCTGAC CTGCGCTGGG CCGCAAGCCG ACGCGCCCTG  
 12181 GAGGACGCTG GGGCCGGACC TGGGCTGGCG GTGGCACCCG CGCGCGCTGG CAACGTCGGC  
 12241 GCGGTGGAGG AATATGACGA GGACGATGAG TACGAGCCAG AGGACGGCGA GTACTAAGCG  
 12301 GTGATGTTTC TGATCAGATG ATGCAAGACG CAACGGACCC GCGGTGCGG GCGGCGCTGC  
 12361 AGAGCCAGCC GTCCGGCCTT AACTCCACGG ACGACTGGCG CCAGGTCATG GACCGCATCA  
 12421 TGTGCTGAC TGCAGCAAT CCTGACGCGT TCGGCGAGCA GCGGCAAGCC CACGACGAG AAGGTGCTGG  
 12481 CCGCAATTCT GGAAGCGGTG GTCCCGGCGC GCGCAAAACC CACGACGAG AAGGTGCTGG  
 12541 CGATCGTAAA CGCGCTGGCC GAAACAGGG CCATCCGGCC CGACGAGGCC GGCCTGGTCT  
 12601 ACGACGCGCT GCTTCAGCGC GTGGCTCGTT ACAACAGCGG CAACGTGCAG ACCAACCTGG  
 12661 ACCGCTGGT GGGGATGTG CGCGAGGCGG TGGCGCAGCG TGAGCGCGCG CAGCAGCAGG  
 12721 GCAACCTGGG CTCCATGGTT GCACTAAACG CCTTCCTGAG TACACAGCCC GCCAACGTGC  
 12781 CGCGGGGACA GGAGGACTAC ACCAACTTTG TGAGCGCACT GCGGCTAATG GTGACTGAGA  
 12841 CACCGCAAAG TGAGGTGTAC CAGTCTGGGC CAGACTATTT TTTCCAGACC AGTAGACAAG  
 12901 GCCTGCAGAC CGTAAACCTG AGCCAGGCTT TCAAAAACCT GCAGGGGCTG TGGGGGGTGC

FIGURE 21  
(SHEET 4)

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12961 GGGCTCCAC AGGCGACCGC GCGACCGTGT CTAGCTTGCT GACGCCAAC TCGCGCCTGT  
13021 TGCTGTGCT AATAGCGCCC TTCACGGACA GTGGCAGCGT GTCCCGGGAC ACATACCTAG  
13081 GTCACCTGCT GACACTGTAC CGCGAGGCCA TAGGTCAGGC GCATGTGGAC GAGCATACTT  
13141 TCCAGGAGAT TACAAGTGTC AGCCGCGCGC TGGGGCAGGA GGACACGGGC AGCCTGGAGG  
13201 CAACCCTAAA CTACCTGCTG ACCAACCCGC GGCAGAAGAT CCCCTCGTTG CACAGTTTAA  
13261 ACAGCGAGGA GGAGCGCAAT TTGCGCTACG TGCAGCAGAG CGTGAGCCTT AACCTGATGC  
13321 GCGACGGGGT AACGCCCAGC GTGGCGCTGG ACATGACCGC GCGCAACATG GAACCGGGCA  
13381 TGTATGCCTC AAACCGGCCG TTTATCAACC GCCTAATGGA CTACTTGCTG CGCGCGGCCG  
13441 CCGTGAACCC CGAGTATTTT ACCAATGCCA TCTTGAACCC GCACTGGCTA CCGCCCCCTG  
13501 GTTCTACAC CGGGGGATTG GAGGTGCCCG AGGGTAACGA TGGATTCTCT TGGGACGACA  
13561 TAGACGACAG CGTGTTTTCC CCGCAACCGC AGACCCTGCT AGAGTTGCAA CAGCGCGAGC  
13621 AGGCAGAGGC GCGCTGCGA AAGGAAAGCT TCCGCAGGCC AAGCAGCTTG TCCGATCTAG  
13681 GCGCTGCGGC CCCGCGGTCA GATGCTAGTA GCCCATTTC AAGCTTGATA GGTCTCTTA  
13741 CCAGCACTCG CACCACCCGC CCGCGCCTGC TGGGCGAGGA GGAGTACCTA AACAACTCGC  
13801 TCGTCGAGCC GCAGCGCGAA AAAAACCTGC CTCCGGCATT TCCCAACAAC GGGATAGAGA  
13861 GCCTAGTGGA CAAGATGAGT AGATGGAAGA CGTACGCGCA GGAGCACAGG GACGTGCCAG  
13921 GCCCGCGCCC GCCCACCCGT CGTCAAAGGC ACGACCGTCA GCGGGGTCTG GTGTGGGAGG  
13981 ACGATGACTC GGCAGACGAC AGCAGCGTCC TGGATTGTTG AGGGAGTGGC AACCCGTTTG  
14041 CGCACCTTCG CCCCAGGCTG GGGAGAATGT TTTAAAAAAA AAAAAGCATG ATGCAAAATA  
14101 AAAAATCAC CAAGGCCATG GCACCGAGCG TTGGTTTTCT TGTATTCCCC TTAGTATGCG  
14161 GCGCGCGGCG ATGTATGAGG AAGGTCTCTC TCCCTCTAC GAGAGTGTGG TGAGCGCGGC  
14221 GCCAGTGGCG GCGGCGCTGG GTTCTCCCTT CGATGCTCCC CTGGACCCGC CGTTTGTGCC  
14281 TCCGCGGTAC CTGCGGCCTA CCGGGGGGAG AAACAGCATC CGTTACTCTG AGTTGGCACC  
14341 CCTATTCGAC ACCACCCGTG TGTACCTGGT GGACAACAAG TCAACGGATG TGGCATCCCT  
14401 GAACTACCAG AACGACCACA GCAACTTTCT GACCACGGTC ATTCAAACA ATGACTACAG  
14461 CCCGGGGGAG GCAAGCACAC AGACCATCAA TCTTGACGAC CGGTGCGACT GGGGCGCGGA  
14521 CCTGAAAACC ATCTGCATA CCAACATGCC AAATGTGAAC GAGTTCATGT TTACCAATAA  
14581 GTTTAAGGCG CGGGTGATGG TGTGCGGCTT GCCTACTAAG GACAATCAGG TGGAGCTGAA  
14641 ATACGAGTGG GTGGAGTTCA CGCTGCCCGA GGGCAACTAC TCCGAGACCA TGACCATAGA  
14701 CCTATGAAC AACCGCATCG TGGAGCACTA CTTGAAAGTG GGCAGACAGA ACGGGGTCTT  
14761 GGAAGCGCAC ATCGGGGTAA AGTTTGACAC CCGCAACTTC AGACTGGGGT TTGACCCCGT  
14821 CACTGGTCTT GTCATGCTG GGGTATATAC AAACGAAGCC TTCCATCCAG ACATCATTTT  
14881 GCTGCCAGGA TGCGGGGTGG ACTTCACCCA CAGCCGCTG AGCAACTTGT TGGGCATCCG  
14941 CAAGCGGCAA CCCTTCCAGG AGGGCTTTAG GATCACCTAC GATGATCTGG AGGGTGGTAA  
15001 CATTCGCGCA CTGTTGGATG TGGACGCCTA CCAGGCGAGC TTGAAAGATG ACACCGAACA  
15061 GGGCGGGGGT GGCGCAGGCG GCAGCAACAG CAGTGGCAGC GCGCGGGAAG AGAACTCCAA  
15121 CGCGCGAGCC GCGGCAATGC AGCCGGTGGA GGACATGAAC GATCATGCCA TTCGCGGCGA  
15181 CACCTTTGCC ACACGGGCTG AGGAGAAGCG CGCTGAGGCC GAAGCAGCGG CCGAAGCTGC  
15241 CGCCCCGCT GCGCAACCCG AGGTGCGAAG GCCTCAGAAG AAACCGGTGA TCAAACCCCT  
15301 GACAGAGGAC AGCAAGAAAC GCAGTTACAA CCTAATAAGC AATGACAGCA CCTTCACCCA  
15361 GTACCGCAGC TGGTACCTTG CATACAATA CGGCGACCTT CAGACCGGAA TCCGCTCATG  
15421 GACCTGCTT TGCATCCTG ACGTAACCTG CGGCTCGGAG CAGGTCTACT GGTCTTGCC  
15481 AGACATGATG CAGACCCCG TGACCTTCCG CTCCACGCGC CAGATCAGCA ACTTTCCGGT  
15541 GGTGGGCGCC GAGCTGTTGC CCGTGCACTC CAAGAGCTTC TACAACGACC AGGCCGTCTA  
15601 CTCCCAACTC ATCCGCCAGT TTACCTCTCT GACCCACGTG TTCAATCGCT TTCCGAGAA  
15661 CCAGATTTTG GCGCGCCCGC CAGCCCCAC CATCACCACC GTCAGTGAAG ACGTTCCTGC  
15721 TCTCACAGAT CACGGGACGC TACCGCTGCG CAACAGCATC GGAGGAGTCC AGCGAGTGAC  
15781 CATTACTGAC GCCAGACGCC GCACCTGCCC CTACGTTTAC AAGGCCCTGG GCATAGTCTC  
15841 GCCGCGCGTC CTATCGAGCC GCACTTTTGG AGCAAGCATG TCCATCCTTA TATCGCCCAG  
15901 CAATAACACA GGTGTTGGCC TGCGCTTCCC AAGCAAGATG TTTGGCGGGG CCAAGAAGCG  
15961 CTTCCGACAA CACCCAGTGC GCGTGCAGG GCACTACCGC GCGCCCTGGG GCGCGCACAA  
16021 ACGCGGCCGC ACTGGGCGCA CCACCGTCGA TGACGCCATC GACGCGGTGG TGGAGGAGGC  
16081 GCGCAACTAC ACGCCACGCG CGCCACCACT GTCCACAGTG GACGCGGCCA TTCAGACCGT  
16141 GGTGCGCGGA GCCCGCGCT ATGCTAAAT GAAGAGACGG CCGAGGCGCG TAGCACGTGC  
16201 CCACCGCCGC CGACCCGGCA CTGCGGCCA ACGCGCGCG GCGGCCCTGC TTAACCGCGC  
16261 ACGTCGACCC GGCCGACGGG CGGCCATGCG AGGCTGGCGG AGGCTGGCGG CCGGTATTGT  
16321 CACTGTGCCC CCCAGGTCCA GGCAGCAGC GGCCTGCGCA GCAGCCGCGG CCATTAGTGC

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16381 TATGACTCAG GGTGCGAGGG GCAACGTGTA TTGGGTGCGC GACTCGGTGA GCGGCCTGCG
16441 CGTGCCCGTG CGCACCCGCC CCCCGCGCAA CTAGATTGCA AGAAAAAACT ACTTAGACTC
16501 GTACTGTTGT ATGTATCCAG CGGCGGCGGC GCGCAACGAA GCTATGTCCA AGCGCAAAAT
16561 CAAAGAAGAG ATGCTCCAGG TCATCGCGCC GGAGATCTAT GGCCCCCGA AGAAGGAAGA
16621 GCAGGATTAC AAGCCCCGAA AGCTAAAGCG GGTCAAAAAG AAAAAGAAAG ATGATGATGA
16681 TGAACCTGAC GACGAGGTGG AACTGCTGCA CGTACCGCG CCCAGGCGAC GGGTACAGTG
16741 GAAAGGTGGA CGCGTAAAC GTGTTTTCG ACCCGGCACC ACCGTAGTCT TTACGCCCGG
16801 TGAGCGCTCC ACCCGCACCT ACAAGCGCGT GTATGATGAG GTGTACGGCG ACGAGGACCT
16861 GCTTGAGCAG GCCAACGAGC GCCTCGGGGA GTTTGCCTAC GGAAAGCGGC ATAAGGACAT
16921 GCTGGCGTTG CCGCTGGACG AGGGCAACCC AACACCTAGC CTAAAGCCCG TAACACTGCA
16981 GCAGGTGCTG CCCGCGCTTG CACCGTCCGA AGAAAAGCGC GGCTAAAGC GCGAGTCTGG
17041 TGACTTGGCA CCCACCGTGC AGCTGATGGT ACCCAAGCGC CAGCGACTGG AAGATGTCTT
17101 GGAAAAAATG ACCGTGGAAC CTGGGCTGGA GCCCGAGGTC CGCGTGCGGC CAATCAAGCA
17161 GGTGGCGCGG GGAAGTGGCG TGCAGACCGT GGACGTTTCA ATACCCACTA CCAGTAGCAC
17221 CAGTATTGCC ACCGCCACAG AGGGCATGGA GACACAAACG TCCCGGTTG CCTCAGCGGT
17281 GCGGATGCC GCGGTGCAGG CGGTGCTGC GCGCGCGTCC AAGACCTCTA CGGAGGTGCA
17341 AACGGACCCG TGGATGTTTC GCGTTTCAGC CCCCGGCGC CCGCGCGGTT CGAGGAAGTA
17401 CCGCGCCGCG AGCGCGCTAC TGCCCGAATA TGCCCTACAT CCTTCCATTG CGCCTACCCC
17461 CGGCTATCGT GGCTACACCT ACCGCCCGAG AAGACGAGCA ACTACCCGAC GCCGAACAC
17521 CACTGGAACC CGCCGCCGCC GTGCGCGTGC CCAGCCCGTG CTGGCCCCGA TTTCGCTGCG
17581 CAGGGTGGCT CGCGAAGGAG GCAGGACCTT GGTGCTGCCA ACAGCGCGCT ACCACCCAG
17641 CATCGTTTAA AAGCCGCTCT TTGTGTTTCT TGCAGATATG GCCCTCACCT GCCGCCTCCG
17701 TTTCCCGGTG CCGGGATTCC GAGGAAGAAT GCACCGTAGG AGGGGCATGG CCGGCCACGG
17761 CCTGACGGGC GGCATGCGTC GTGCGCACCA CCGCGGCGG CGCGCGTGC ACCGTGCGAT
17821 GCGCGGCGGT ATCCTGCCCT TCCTTATTCC ACTGATCGCC GCGGCGATTG GCGCGGTGCC
17881 CGGAATTGCA TCCGTGGCCT TGCAGGCGCA GAGACACTGA TTA AAAACAA GTTGATGTG
17941 GAAAAATCAA AATAAAAAGT CTGGACTCTC ACGCTCGCTT GGTCTGTAA CTATTTTGTA
18001 GAATGGAAGA CATCAACTTT GCGTCTCTGG CCCCGCGACA CGGCTCGCGC CCGTTCATGG
18061 GAAACTGGCA AGATATCGGC ACCAGCAATA TGAGCGGTGG CGCCTTCAGC TGGGGCTCGC
18121 TGTTGGAGCG CATTAAAAAT TTCGGTTCCA CCGTTAAGAA CTATGGCAGC AAGGCTGGA
18181 AGCAGCAC AGGCCAGATG CTGAGGGATA AGTTGAAAGA GCAAAATTTC CAACAAAGG
18241 TGGTAGATGG CCTGGCCTCT GGCATTAGCG GGGTGGTGGG CCTGGCCAAC CAGGCAGTGC
18301 AAAATAAGAT TAACAGTAAG CTTGATCCCC GCCCTCCCGT AGAGGAGCCT CCACCGCGCG
18361 TGGAGACAGT GTCTCCAGAG GGGCGTGGCG AAAAGCGTCC GCGCCCCGAC AGGGAAGAAA
18421 CTCTGGTGAC GCAAATAGAC GAGCCTCCCT CGTACGAGGA GGCACTAAG CAAGGCCTGC
18481 CCACCACCCG TCCATCGCG CCCATGGCTA CCGGAGTGCT GGGCCAGCAC ACACCCGTAA
18541 CGCTGGACCT GCGTCCCCC CCGGACACCC AGCAGAAACC TGTGCTGCCA GGCCCCGACG
18601 CCGTTGTTGT AACCCGTCCT AGCCGCGCGT CCCTGCGCG CGCCGCCAGC GGTCCGCGAT
18661 CGTTGCGGCC CGTAGCCAGT GGCAACTGGC AAAGCACACT GAACAGCATC GTGGGTCTGG
18721 GGGTGCAATC CCTGAAGCGC CGACGATGCT TCTGAATAGC TAACGTGTGC TATGTGTGTC
18781 ATGTATGCGT CCATGTGCGC GCCAGAGGAG CTGCTGAGCC GCGCGCGGCC CGCTTTCCAA
18841 GATGGCTACC CCTTCGATGA TGCCGAGTG GTCTTACATG CACATCTCGG GCCAGGACGC
18901 CTCGGAGTAC CTGAGCCCCG GGCTGTGTGA GTTTGCCCGC GCCACCGAGA CGTACTTCAG
18961 CCTGAATAAC AAGTTTAGAA ACCCCACGGT GCGGCTACG CACGACGTGA CCACAGACCG
19021 GTCCAGCGT TTGACGCTGC GGTTCATCCC TGTGACCGT GAGGATACTG CGTACTCGTA
19081 CAAGGCGCGG TTCACCCTAG CTGTGGGTGA TAACCGTGTG CTGGACATGG CTTCCACGTA
19141 CTTTGACATC CGCGGCGTGC TGGACAGGGG CCCTACTTTT AAGCCCTACT CTGGCACTGC
19201 CTACAACGCC CTGGCTCCA AGGGTGCCCC AAATCCTTGC GAATGGGATG AAGCTGCTAC
19261 TGCTCTTGAA ATAAACCTAG AAGAGAGGA CGATGACAAC GAAGACGAAG TAGACGAGCA
19321 AGCTGAGCAG CAAAAAACTC ACGTATTTGG GCAGGCGCCT TATTCTGGTA TAAATATTAC
19381 AAGGAGGGT ATTCAAATAG GTGTGGAAGG TCAACACCT AAATATGCCG ATAAACATT
19441 TCAACCTGAA CCTCAAATAG GAGATCTCA GTGGTACGAA ACTGAAATTA ATCATGCAGC
19501 TGGGAGAGTC CTTAAAAAGA CTACCCCAAT GAAACCATGT TACGGTTCAT ATGCAAAACC
19561 CACAAATGAA AATGGAGGCG AAGGCATTCT TGTAAAGCA CAAAATGGAA AGCTAGAAAG
19621 TCAAGTGGAA ATGCAATTTT TCTCAACTAC TGAGGCGACC GCAGGCAATG GTGATAACTT
19681 GACTCCTAAA GTGGTATTGT ACAGTGAAGA TGTAGATATA GAAACCCAG ACACTCATAT
19741 TTCTTACATG CCCACTATTA AGGAAGGTAA CTCACGAGAA CTAATGGGCC AACAATCTAT

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19801 GCCCAACAGG CCTAATTACA TTGCTTTTAG GGACAATTTT ATTGGTCTAA TGTATTACAA  
19861 CAGCACGGGT AATATGGGTG TTCTGCGGG CCAAGCATCG CAGTTGAATG CTGTTGTAGA  
19921 TTGCAAGAC AGAAACACAG AGCTTTCATA CCAGCTTTTG CTTGATTCCA TTGGTGATAG  
19981 AACCAGGTAC TTTTCTATGT GGAATCAGGC TGTGACAGC TATGATCCAG ATGTTAGAAT  
20041 TATTGAAAAT CATGGAACTG AAGATGAACT TCCAAATTAC TGCTTTCCAC TGGGAGGTGT  
20101 GATTAATACA GAGACTCTTA CCAAGGTAAA ACCTAAACA GGTCAAGAAA ATGGATGGGA  
20161 AAAAGATGCT ACAGAATTTT CAGATAAAAA TGAAATAAGA GTTGGAATA ATTTTGCCAT  
20221 GGAAATCAAT CTAAATGCCA ACCTGTGGAG AAATTTCCCTG TACTCCAACA TAGCGCTGTA  
20281 TTGCCCAGAC AAGCTAAAGT ACAGTCTTTC CAACGTAAAA ATTTCTGATA ACCCAAACAC  
20341 CTACGACTAC ATGAACAAGC GAGTGGTGGC TCCCGGGTTA GTGGACTGCT ACATTAACTT  
20401 TGGAGCACGC TGGTCCCTTG ACTATATGGA CAACGTCAAC CCATTTAACC ACCACCGCAA  
20461 TGCTGGCCTG CGCTACCGCT CAATGTTGCT GGGCAATGGT CGCTATGTGC CCTTCCACAT  
20521 CCAGGTGCCT CAGAAGTTCT TTGCCATTAA AAACCTCCTT CTCCTGCCGG GCTCATACAC  
20581 CTACGAGTGG AACTTCAGGA AGGATGTTAA CATGGTTCTG CAGAGCTCCC TAGGAAATGA  
20641 CCTAAGGGTT GACGGAGCCA GCATTAAAGT TGATAGCATT TGCTTTTACG CCACCTTCTT  
20701 CCCCATGGCC CACAACACCG CCTCCACGCT TGAGGCCATG CTTAGAAACG ACACCAACGA  
20761 CCAGTCCCTT AACGACTATC TCTCCGCGC CAACATGCTC TACCCTATAC CCGCCAACGC  
20821 TACCAACGTG CCCATATCCA TCCCCCTCCG CAACTGGGCG GCTTTCCGCG GCTGGGCTT  
20881 CACGCGCCTT AAGACTAAGG AAACCCATC ACTGGGCTCG GGCTACGACC CTTATTACAC  
20941 CTACTCTGGC TCTATACCTT ACCTAGATGG AACCTTTTAC CTCAACCACA CTTTAAAGAA  
21001 GGTGGCCATT ACCTTTGACT CTTCTGTGAG CTGGCCTGGC AATGACCGCC TGCTTACCCC  
21061 CAACGAGTTT GAAATTAAGC GCTCAGTTGA CGGGGAGGGT TACAACGTG CCCAGTGATA  
21121 CATGACCAAA GACTGGTTCC TGGTACAAAT GCTAGCTAAC TACAACATTG GCTACCAGGG  
21181 CTTCTATATC CCAGAGAGCT ACAAGGACCG CATGTACTCC TTCTTTAGAA ACTTCCAGCC  
21241 CATGAGCCGT CAGGTGGTGG ATGATACTAA ATACAAGGAC TACCAACAGG TGGGCATCCT  
21301 ACACCAACAC AACAACCTG GATTTGTTGG CTACCTTGCC CCCACCATGC GCGAAGGACA  
21361 GGCCTACCCT GCTAACTTCC CCTATCCGCT TATAGGCAAG ACCGCAGTTG ACAGCATTAC  
21421 CCAGAAAAAG TTTCTTTGCG ATCGCACCTT TTGGCGCATC CCATTCTCCA GTAACCTTAT  
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21541 GCTAGACATG ACTTTTGAGG TGGATCCCAT GGACGAGCCC ACCCTTCTTT ATGTTTGTGTT  
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21721 ACAGCTGCCG CCATGGGCTC CAGTGAGCAG GAACTGAAAG CCATTGTCAA AGATCTGGT  
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21841 AAGCTCGCCT GCGCCATAGT CAATACGGCC GGTGCGGAGA CTGGGGGCGT AACTGGATG  
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21961 GAACGACGAC TCAAGCAGGT TTACCGATT TTAGCAGAGT CACTCCTGCG CCGTAGCGCC  
22021 ATTGCTTCTT CCCCCGACCG CTGTATAACG CTGAAAAGT CCACCCAAAG CGTACAGGGG  
22081 CCCAACTCGG CCGCCTGTGG ACTATTCTGC TGCATGTTTC TCCACGCCTT TGCCAACTGG  
22141 CCCCAACTC CCATGGATCA CAACCCACC ATGAACCTTA TTACCGGGGT ACCCAACTCC  
22201 ATGCTCAACA GTCCCCAGGT ACAGCCCACC CTGCGTCGCA ACCAGGAACA GCTCTACAGC  
22261 TTCTGGAGC GCCACTCGCC CTACTTCCG AGCCACAGT GCGAGATTAG GAGCGCCACT  
22321 TCTTTTGTG ACTTGAAAAA CATGTAATAA TAATGTACTA GAGACACTTT CAATAAAGGC  
22381 AAATGCTTTT ATTTGTACAC TCTCGGGTGA TTATTTACCC CCACCCTTGC CGTCTGCGCC  
22441 GTTTAAAAAT CAAAGGGGTT CTGCCGCGCA TCGCTATGCG CCACTGGCAG GGACACGTG  
22501 CGATACTGGT GTTTAGTGCT CCACTTAAAC TCAGGCACAA CCATCCGCGG CAGCTCGGTG  
22561 AAGTTTTTAC TCCACAGGCT GCGCACCATC ACCAACGCGT TTAGCAGGTC GGGCGCGGAT  
22621 ATCTTGAAGT CGCAGTTGGG GCCTCCGCCC TGCGCGCGCG AGTTGCGATA CACAGGGTTG  
22681 CAGCACTGGA ACACTATCAG CGCCGGGTGG TGACGCTGG CCAGCACGCT CTTGTGCGAG  
22741 ATCAGATCCG CGTCCAGGTC CTCGCGGTG CTCAGGGCGA ACGGAGTCAA CTTTGGTAGC  
22801 TGCTTCCCA AAAAGGGGCG GTGCCAGGC TTTGAGTTGC ACTCGCACCG TAGTGGCATC  
22861 AAAAGGTGAC CGTGCCCGGT CTGGGCGTTA GGATACAGCG CCTGCATAAA AGCCTTGATC  
22921 TGCTTAAAG CCACCTGAGC CTTTGCGCCT TCAGAGAAGA ACATGCCGCA AGACTTGGCG  
22981 GAAAAGTATG TGGCCGGACA GGCCGCGTCG TGCAACGAGC ACCTTGCGTC GGTGTTGGAG  
23041 ATCTGCACCA CATTTCGGCC CCACCGGTTT TTCAGATCT TGGCCTTGCT AGACTGCTCC  
23101 TTCAGCGCGC GCTGCCCGTT TTCGCTCGTC ACATCCATTT CAATCAGGTG CTCCTTATTT  
23161 ATCATAATGC TTCCGTGTAG ACACCTTAAGC TCGCTTCGA TCTCAGCGCA GCGGTGCAGC



23221 CACAACGCGC AGCCCGTGGG CTCGTGATGC TTGTAGGTCA CCTCTGCAAA CGACTGCAGG  
 23281 TACGCCTGCA GGAATCGCCC CATCATCGTC ACAAAGGTCT TGTGTCTGGT GAAGGTGAGC  
 23341 TGCAACCCGC GGTGCTCCTC GTTCAGCCAG GTCTTGACATA CGGCCGCCAG AGCTTCCACT  
 23401 TGGTCAGGCA GTAGTTTGAA GTTCGCCTTT AGATCGTTAT CCACGTGGTA CTTGTCCATC  
 23461 AGCGCGCGCG CAGCCTCCAT GCCCTTCTCC CACGCAGACA CGATCGGCAC ACTCAGCGGG  
 23521 TTCATCACCG TAATTTCACT TTCCGCTTCG CTGGGCTCTT CCTCTTCCTC TTGCGTCCGC  
 23581 ATACCACGCG CCACTGGGTC GTCTTCATT AGCCGCGGCA CTGTGCGCTT ACCTCCTTTG  
 23641 CCATGCTTGA TTAGCACCGG TGGGTTGCTG AAACCCACCA TTTGTAGCGC CACATCTTCT  
 23701 CTTTCTTCCT CGCTGTCCAC GATTACCTCT GGTGATGGCG GCGGCTCGGG CTTGGGAGAA  
 23761 GGGCGCTTCT TTTTCTTCTT GGGCGCAATG GCCAAATCCG COGCCGAGGT CGATGCCCCG  
 23821 GGGCTGGGTG TGC CGGCAC CAGCGCGTCT TGTGATGAGT CTTCTCTGTC CTCGACTCG  
 23881 ATACGCGGCC TCATCCGCTT TTTTGGGGGC GCCCGGGGAG GCGGCGGCGA CGGGGACGGG  
 23941 GACGACACGT CCTCCATGGT TGGGGGACGT CGCGCGGCAC CGCGTCCGCG CTCGGGGGTG  
 24001 GTTTCGCGCT GCTCCTCTTC CCGACTGGCC ATTTCTTCTT CCTATAGGCA GAAAAAGATC  
 24061 ATGGAGTCAG TCGAGAAGAA GGACAGCCTA ACCGCCCCCT CTGAGTTCGC CACCACCGCC  
 24121 TCCACCGATG CCGCCAACGC GCCTACCACC TTCCCGTCTG AGGCACCCCC GCTTGAGGAG  
 24181 GAGGAAGTGA TTATCGAGCA GGACCCAGGT TTTGTAGCGG AAGACGACGA GGACCGCTCA  
 24241 GTACCAACAG AGGATAAAAA GCAAGACCAG GACAACGCGC AGGCAAAACGA GGAACAAGTC  
 24301 GGGCGGGGGG ACGAAAGGCA TGGCGACTAC CTAGATGTGG GAGACGACGT GCTGTGGAAG  
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 24541 TTTGCCGTGC CAGAGGTGCT TGCCACCTAT CACATCTTTT TCCAAACTG CAAGATACCC  
 24601 CTATCCTGCC GTGCCAACCG CAGCCGAGCG GACAAGCAGC TGGCCTTGCG GCAGGCGCT  
 24661 GTCATACCTG ATATCGCCTC GCTCAACGAA GTGCCAAAAA TCTTTGAGGG TCTTGAGCGC  
 24721 GTCGAGAAGC GCGCGGCAAA CGCTCTGCAA CAGGAAAAA GCGAAAATGA AAGTCACTCT  
 24781 GGAGTGTGGG TGGAATCGA GGGTGACAAC GCGCGCCTAG CCGTACTAAA ACGCAGCATC  
 24841 GAGGTCAACC ACTTTGCCTA CCGGCACTT AACCTACCCC CCAAGGTCTAT GAGCAGATC  
 24901 ATGAGTGAGC TGATCGTGCG CCGTGCGCAG CCCCTGGAGA GGGATGCAAA TTTGCAAGAA  
 24961 CAAACAGAGG AGGGCCTACC CGCAGTTGGC GACGAGCAGC TAGCGCGCTG GCTTCAAACG  
 25021 CGCGAGCCTG CCGACTTGGG GGAGCGAGCG AAATAATGA TGGCCGCGAGT GCTCGTTACC  
 25081 GTGGAGCTTG AGTGTCATGA GCGTTCTTTT GCTGACCCGG AGATGCAGCG CAAGCTAGAG  
 25141 GAAACATTGC ACTACACCTT TCGACAGGGC TACGTACGCC AGGCCTGCAA GATCTCCAAC  
 25201 GTGGAGCTCT GCAACCTGGT CTCTACCTT GGAATTTTGC ACGAAAACCG CCTTGGGCAA  
 25261 AACGTGCTTC ATTCCACGCT CAAGGGCGAG GCGCGCCGCG ACTACGTCCG CGACTGCGTT  
 25321 TACTTATTTT TATGCTACAC CTGGCAGACG GCCATGGGCG TTTGGCAGCA GTGCTTGAGG  
 25381 GAGTGCAACC TCAAGGAGCT GCAGAAACATG CTAAAGCAAA ACTTGAAGGA CCTATGGACG  
 25441 GCCTTCAACG AGCGCTCCGT GGCCGCGCAC CTGGCGGACA TCATTTTCCC CGAACGCTG  
 25501 CTTAAACCCC TGCAACAGGG TCTGCCAGAC TTCACCAGTC AAAGCATGTT GCAGAACTTT  
 25561 AGGAACTTTA TCCTAGAGCG CTCAGGAATC TTGCCCGCCA CCTGCTGTGC ACTTCTTAGC  
 25621 GACTTTGTGC CCATTAAGTA CCGCGAATGC CCTCCGCGCG TTTGGGGCCA CTGCTACCTT  
 25681 CTGCAGCTAG CCAACTACCT TGCCCTACCAC TCTGACATAA TGGAAGACGT GAGCGGTGAC  
 25741 GGTCTACTTG AGTGTCACTG TCGCTGCAAC CTATGCACCC CGCACCGCTC CCTGTTTTCG  
 25801 AATTGCGAGC TGCTTAACGA AAGTCAAATT ATCGGTACCT TTGAGCTGCA GGGTCCCTCG  
 25861 CCTGACGAAA AGTCCGCGGC TCCGGGGTTG AAACCTACTC CGGGGCTGTG GACGTGCGCT  
 25921 TACCTTCGCA AATTTGTACC TGAGGACTAC CACGCCCACG AGATTAGGTT CTACGAAGAC  
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 26041 GGCCAATTGC AAGCCATCAA CAAAGCCCGC CAAGAGTTTC TGCTACGAAA GGGACGGGGG  
 26101 GTTACTTTGG ACCCCAGTTC CGGCGAGGAG CTCAACCCAA TCCCCCGCC GCCGCGAGCC  
 26161 TATCAGCAGC AGCCGCGGGC CTTGCTTCC CAGGATGGCA CCCAAAAAGA AGCTGCAGCT  
 26221 GCGCGCGCCA CCCACGGACG AGGAGGAATA CTGGGACAGT CAGGCGAGAG AGGTTTTTGA  
 26281 CGAGGAGGAG GAGGACATGA TGGAAGACTG GGAGAGCCTA GACGAGGAAG CTTCCGAGGT  
 26341 CGAAGAGGTG TCAGACGAAA CACCGTACC CTGGTTCGCA TTCCCTTCGC CGCGGCCCA  
 26401 GAAATCGGCA ACCGTTTCCA GCATGGCTAC AACCTCCGCT CCTCAGGCGC CGCCGCACT  
 26461 GCCCGTTCCG CAGCCCAACC GTAGATGGGA CACCACTGGA ACCAGGGCCG GTAAGTCCAA  
 26521 GCAGCGCGCG CCGTTAGCCC AAGAGCAACA ACAGCGCCAA GGCTACCGCT CATGGCGCGG  
 26581 GCACAAGAAC GCCATAGTTG CTTGCTTGCA AGACTGTGGG GGCAACATCT CTTTCGCGCG

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26641 CCGCTTTCTT CTCTACCATC ACGGCGTGCC CTTCCCCCGT AACATCCTGC ATTACTACCG
26701 TCATCTCTAC AGCCCATACT GCACGGGCGG CAGCGGCAGC GGCAGCAACA GCAGCGGCCA
26761 CACAGAAGCA AAGGCGACCG GATAGCAAGA CTCTGACAAA GCCCAAGAAA TCCACAGCGG
26821 CGGCAGCAGC AGGAGGAGGA GCGCTGCGTC TGGCGCCCAA CGAACCCGTA TCGACCCGCG
26881 AGCTTAGAAA CAGGATTTT CCCACTCTGT ATGCTATATT TCAACAGAGC AGGGGCCAAG
26941 AACAAGAGCT GAAAAATAAA AACAGGTCTC TGCATCCCT CACCCGCGAGC TGCCTGTATC
27001 ACAAAGCGA AGATCAGCTT CGGCGCACGC TGAAGACGC GGAGGCTCTC TTCAGTAAAT
27061 ACTGCGCGCT GACTCTTAAG GACTAGTTTC GCGCCCTTTC TCAAATTTAA GCGCGAAAAC
27121 TACGTCTATCT CCAGCGGCCA CACCCGCGGC CAGCACCTGT CGTCAGCGCC ATTATGAGCA
27181 AGGAAATTCC CACGCCCTAC ATGTGGAGTT ACCAGCCACA AATGGGACTT GCGGCTGGAG
27241 CTGCCCAAGA CTACTCAACC CGAATAAACT ACATGAGCGC GGGACCCAC ATGATATCCC
27301 GGGTCAACCG AATCCGCGCC CACCGAAACC GAATTCTCTT GGAACAGGCG GCTATTACCA
27361 CCACACCTCG TAATAACCTT AATCCCCGTA GTTGGCCCGC TGCCCTGGTG TACCAGGAAA
27421 GTCCCGCTCC CACCACTGTG GTACTTCCCA GAGACGCCCA GGCCGAAGTT CAGATGACTA
27481 TCTCAGGGGC GCAGCTTGGC GCGGCTTTTC GTCACAGGGT GCGGTCGCC GGGCAGGGTA
27541 TAACTCACCT GACAATCAGA GGGCGAGGTA TTCAGCTCAA CGACGAGTCG GTGAGCTCCT
27601 CGCTTGGTCT CCGTCCGAC GGGACATTTT AGATCGGCGG CGCCGCGCGT CCTTCATTCA
27661 CGCCTCGTCA GGCAATCTTA ACTCTGCAGA CCTCGTCTC TGAGCCGCGC TCTGGAGGCA
27721 TTGGAATCTT GCAATTTATT GAGGAGTTTG TGCCATCGGT CTACTTTAAC CCCTTCTCGG
27781 GACCTCCCGG CCACCTATCCG GATCAATTTA TTCCTAAGTT TGACGCGGTA AAGGACTCGG
27841 CGGACGGCTA CGACTGAATG TTAAGTGGAG AGGCAGAGCA ACTGCGCCTG AAACACCTGG
27901 TCCACTGTG CCGCCACAAG TGCTTTGCC GCGACTCCGG TGAGTTTTC TACTTTGAAT
27961 TGCCCGAGGA TCATATCGAG GGCCCGGCGC ACGGCGTCCG GCTTACCGCC CAGGAGAGC
28021 TTGCCCCGTA CTGATTCGG GAGTTTACCC AGCGCCCCCT GCTAGTTGAG CGGGACAGGG
28081 GACCCTGTGT TCTCACTGTG ATTTGCAACT GTCCTAACCT TGGATTACAT CAAGATCTTT
28141 GTTGCCATCT CTGTGCTGAG TATAATAAAT ACAGAAATTA AAATATACTG GGGCTCCTAT
28201 CGCCATCCTG TAAACGCCAC CGTCTTACC CGCCCAAGCA AACCAGGCG AACCTTACCT
28261 GGTACTTTTA ACATCTCTCC CTCTGTGATT TACAACAGT TCAACCCAGA CGGAGTGAGT
28321 CTACGAGAGA ACCTCTCGA GCTCAGCTAC TCCATCAGAA AAAACACCAC CCTCCTTACC
28381 TGCCGGGAAC GTACGAGTGC GTCACCGGCC GCTGCACCAC ACCTACCGCC TGACCGTAAA
28441 CCAGACTTTT TCCGGACAGA CCTCAATAAC TCTGTTTACC AGAACAGGAG GTGAGCTTAG
28501 AAAACCTTTA GGGTATTAGG CCAAAGGCGC AGCTACTGTG GGGTTTATGA ACAAATCAAG
28561 CAACTCTACG GGCTATTCTA ATTCAGGTTT CTCTAGAATC GGGGTGGGG TTATTCTCTG
28621 TCTGTGATT CTCTTTATTC TTATACTAAC GCTTCTCTGC CTAAGGCTCG CCGCTGCTG
28681 TGTGCACATT TGCAATTATT GTCAGCTTTT TAAACGCTGG GGTCCGCCACC CAAGATGATT
28741 AGGTACATAA TCCTAGGTTT ACTACCCCTT GCGTCAGCCC ACGGTACCAC CCAAAGGTG
28801 GATTTTAAAG AGCCAGCCTG TAATGTTACA TTCGAGCTG AAGCTAATGA GTGCACCATT
28861 CTTATAAAT GCACCACAGA ACATGAAGA CTGCTTATTC GCCACAAAAA CAAATTTGGC
28921 AAGTATGCTG TTTATGCTAT TTGGCAGCCA GGTGACACTA CAGAGTATAA TGTACAGTT
28981 TTCCAGGGTA AAAGTCATAA AACTTTTATG TATACTTTTC CATTTTATGA AATGTGCGAC
29041 ATTACCATGT ACATGAGCAA ACAGTATAAG TTGTGGCCCC CACAAATTTG TGTGAAAAAC
29101 ACTGGCACTT TCTGTGCAC TGCTATGCTA ATTACAGTGC TCGCTTTGGT CTGTACCCTA
29161 CTCTATATTA AATACAAAAG CAGACGCGAG TTTATTGAGG AAAAGAAAAT GCCTTAATTT
29221 ACTAAGTTAC AAAGCTAATG TCACCACTAA CTGCTTTACT CGCTGCTTGC AAAACAAATT
29281 CAAAAAGTTA GCATTATAAT TAGAATAGGA TTTAAACCCC CCGGTCAATT CTGTCTCAAT
29341 ACCATTCCCC TGAACAATTG ACTCTATGTG GGATATGCTC CAGCGCTACA ACCTTGAAGT
29401 CAGGCTTCCT GGATGTCAGC ATCTGACTTT GGCCAGCACC TGTCCCGCGG ATTTGTTCCA
29461 GTCCAACCTA AGCGACCCAC CCTAACAGAG ATGACCAACA CAACCAACGC GGC CGCGCT
29521 ACCGGACTTA CATCTACCAC AAATACACCC CAAGTTTCTG CCTTTGTCAA TAACTGGGAT
29581 AACTTGGGCA TGTGGTGGTT CTCCATAGCG CTTATGTTTG TATGCCCTAT TATTATGTGG
29641 CTCATCTGCT GCCTAAAGCG CAAACGCGCC CGACCACCA TCTATAGTCC CATCATGTG
29701 CTACCCCAA ACAATGATGG AATCCATAGA TTGGACGGAC TGAAACACAT GTTCTTTTCT
29761 CTTACAGTAT GATTAAATGA GACATGATTC CTCGAGTTT TATATTACTG ACCCTTGTG
29821 CGCTTTTTC TGCGTGCTCC ACATGGCTC CGGTTTCTCA CATCGAAGTA GACTGCATT
29881 CAGCCTTCAC AGTCTATTG CTTTACGGAT TTGTCACCCT CACGCTCATC TGCAGCTCA
29941 TCACTGTGGT CATCGCCTTT ATCCAGTGCA TTGACTGGGT CTGTGTGCGC TTTGCATATC
30001 TCAGACACCA TCCCCAGTAC AGGACAGGA CTATAGCTGA GCTTCTTAGA ATTCTTTAAT

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30061 TATGAAATTT ACTGTGACTT TTCTGCTGAT TATTTGCACC CTATCTGCGT TTTGTTCCCC  
30121 GACCTCCAAG CCTCAAAGAC ATATATCATG CAGATTCACT CGTATATGGA ATATTCCAAG  
30181 TTGCTACAAT GAAAAAAGCG ATCTTTCCGA AGCCTGGTTA TATGCAATCA TCTCTGTTAT  
30241 GGTGTTCTGC AGTACCATCT TAGCCCTAGC TATATATCCC TACCTTGACA TTGGCTGGAA  
30301 ACGAATAGAT GCCATGAACC ACCCAACTTT CCCC CGCCCC GCTATGCTTC CACTGCAACA  
30361 AGTTGTTGCC GCGGCTTTG TCCCAGCCAA TCAGCCTCGC CCCACTTCTC CCACCCCCAC  
30421 TGAAATCAGC TACTTTAATC TAACAGGAGG AGATGACTGA CACCCTAGAT CTAGAAATGG  
30481 ACGGAATTAT TACAGAGCAG CGCCTGCTAG AAAGACGCAG GGCAGCGGCC GAGCAACAGC  
30541 GCATGAATCA AGAGCTCCAA GACATGGTTA ACTTGCACCA GTGCAAAAGG GGTATCTTTT  
30601 GTCTGGTAAA GCAGGCCAAA GTCACCTACG ACAGTAATAC CACCGGACAC CGCCTTAGCT  
30661 ACAAGTTGCC AACCAAGCGT CAGAAATTGG TGGTCATGGT GGGAGAAAAG CCCATTACCA  
30721 TAACCTCAGCA CTCGGTAGAA ACCGAAGGCT GCATTCACTC ACCTTGTCAG GGACCTGAGG  
30781 ATCTCTGCAC CCTTATTAAG ACCCTGTGCG GTCTCAAAGA TCTTATTCCC TTAACTAAT  
30841 AAAAAAAT AATAAGCAT CACTTACTTA AAATCAGTTA GCAAATTTCT GTCCAGTTTA  
30901 TTCAGCAGCA CCTCCTTGCC CTCCTCCCAG CTCTGGTATT GCAGCTTCTC CTGGCTGCA  
30961 AACTTTCTCC ACAATCTAAA TGGAAATGTCA GTTCTCTCCT GTTCTGTGCC ATCCGCACCC  
31021 ACTATCTTCA TGTGTTGCA GATGAAGCGC GCAAGACCGT CTGAAGATAC CTTCAACCCC  
31081 GTGTATCCAT ATGACACGGA AACCGGTCCCT CCAACTGTGC CTTTTCTTAC TCCTCCCTTT  
31141 GTATCCCCCA ATGGGTTTCA AGAGAGTCCC CCTGGGGTAC TCTCTTTGCG CCTATCCGAA  
31201 CCTCTAGTTA CCTCCAATGG CATGCTTGCG CTCAAAATGG GCAACGGCCT CTCTCTGGAC  
31261 GAGGCCGGCA ACCTTACCTC CCAAAATGTA ACCACTGTGA GCCCACTCTT CAAAAAACC  
31321 AAGTCAAAAC TAAACCTGGA AATATCTGCA CCCCTCACAG TTACCTCAGA AGCCCTAACT  
31381 GTGGCTGCCG CCGCACCTCT AATGGTCGCG GGCAACACAC TCACCATGCA ATCAGAGGCC  
31441 COGCTAACCG TGCACGACTC CAAACTTAGC ATTGCCACCC AAGGACCCCT CACAGTGTCA  
31501 GAAGGAAAGC TAGCCCTGCA AACATCAGGC CCCCTCACCA CCACCGATAG CAGTACCCTT  
31561 ACTATCACTG CCTACCCCC TCTAACTACT GCCACTGGTA GCTTGGGCAT TGACTTGAAA  
31621 GAGCCCATTT ATACACAAAA TGGAAAACTA GGACTAAAGT ACGGGGCTCC TTTGCATGTA  
31681 ACAGACGACC TAAACACTTT GACCGTAGCA ACTGGTCCAG GTGTGACTAT TAATAATACT  
31741 TCCTTGCAAA CTAAAGTTAC TGGAGCCTTG GGTTTTGATT CACAAGGCAA TATGCAACTT  
31801 AATGTACGAG GAGGACTAAG GATTGATTCT CAAAACAGAC GCCTTATACT TGATGTTAGT  
31861 TATCCGTTTG ATGCTCAAAA CCAACTAAAT CTAAGACTAG GACAGGGCCC TCTTTTATA  
31921 AACTCAGCCC ACAACTTGGA TATTAECTAC AACAAAGGCC TTTACTTGTT TACAGCTTCA  
31981 AACAAATCCA AAAAGCTTGA GGTAAACCTA AGCACTGCCA AGGGGTGAT GTTTGACGCT  
32041 ACAGCCATGC CCATTAATGC AGGAGATGGG CTTGAATTTG GTTCACTAA TGCACCAAAC  
32101 ACAAAATCCC TCAAAACAAA AATTGGCCAT GGCCTAGAAT TTGATTCAAA CAAGGCTATG  
32161 GTTCTTAAAC TAGGAACTGG CCTTAGTTTT GACAGCACAG GTGCCATTAC AGTAGGAAAC  
32221 AAAAATAATG ATAAGCTAAC TTTGTGGACC ACACCAGCTC CATCTCTTAA CTGTAGACTA  
32281 AATGCAGAGA AAGATGCTAA ACTCACTTTG GTCTTAACAA AATGTGGCAG TCAAATACTT  
32341 GCTACAGTTT CAGTTTGGC TGTAAAGGC AGTTTGGCTC CAATATCTGG AACAGTTCAA  
32401 AGTGCTCATC TTATTATAAG ATTTGACGAA AATGGAGTGC TACTAAACAA TTCCTTCTTG  
32461 GACCCAGAAT ATTGGAACCT TAGAAATGGA GATCTTACTG AAGGCACAGC CTATACAAAC  
32521 GCTGTTGGAT TTATGCCTAA CCTATCAGCT TATCCAAAAT CTCACGGTAA AACTGCCAAA  
32581 AGTAACATTG TCAGTCAAGT TTACTTAAAC GGAGACAAAA CTAAACCTGT AACACTAACC  
32641 ATTACACTAA ACGGTACACA GGAAACAGGA GACACAACCTC CAAGTGATA CTCTATGTCA  
32701 TTTTCATGGG ACTGGTCTGG CCACAACCTAC ATTAATGAAA TATTTGCCAC ATCCTCTTAC  
32761 ACTTTTTTAT ACATTGCCCC AGAATAAAGA ATCGTTTGTG TTATGTTTCA ACGTGTTTAT  
32821 TTTTCAATTG CAGAAAATTT CAAGTCATTT TTCATTCACT AGTATAGCCC CACCACCACA  
32881 TAGCTTATAC AGATCACCGT ACCTTAATCA AACTCACAGA ACCCTAGTAT TCAACCTGCC  
32941 ACCTCCCTCC CAACACACAG AGTACACAGT CCTTCTCTCC CGGCTGGCCT TAAAAAGCAT  
33001 CATATCATGG GTAACAGACA TATTCTTAGG TGTATATTC CACACGGTTT CCTGTGAGC  
33061 CAAACGCTCA TCAGTGATAT TAATAAACTC CCCGGGCAGC TCACTTAAGT TCATGTGCGT  
33121 GTCCAGCTGC TGAGCCACAG GCTGCTGTCC AACTTGCAGT TGCTTAAACGG GCGGCGAAGG  
33181 AGAAGTCCAC GCCTACATGG GGGTAGAGTC ATAATCGTGC ATCAGGATAG GCGGTTGGTG  
33241 CTGCAGCAGC GCGCGAATAA ACTGCTGCCG CCGCCGCTCC GTCCTGCAGG AATACAACAT  
33301 GGCAGTGGTC TCCTCAGCGA TGATTGCGAC CGCCCGCAGC ATAAGGCGCC TTGTCTCCG  
33361 GGCACAGCAG CGCACCTGTA TCTCACTTAA ATCAGCACAG TAACTGCAGC ACAGCACCAC  
33421 AATATTGTTT AAAATCCCAC AGTGCAAGGC GCTGTATCCA AAGCTCATGG CGGGGACCAC

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33481 AGAACCCACG TGGCCATCAT ACCACAAGCG CAGGTAGATT AAGTGGCGAC CCCTCATAAA
33541 CACGCTGGAC ATAAACATTA CCTCTTTTGG CATGTTGTAA TTCACCACCT CCCGGTACCA
33601 TATAAACCTC TGATTAAACA TGGCGCCATC CACCACCATC CTAAACCAGC TGGCCAAAAC
33661 CTGCCCCCGG GCTATACACT GCAGGGAACC GGGACTGGAA CAATGACAGT GGAGAGCCCA
33721 GGA CTCTGTA CCATGATCA TCATGCTCGT CATGATATCA ATGTTGGCAC AACACAGGCA
33781 CACGTGCATA CACTTCCTCA GGATTACAAG CTCCTCCCGC GTTAGAACCA TATCCAGGG
33841 AACAAACCAT TCCTGAATCA GCGTAAATCC CACACTGCAG GGAAGACCTC GCACGTAACT
33901 CACGTTGTGC ATTGTCAAAG TGTTACATTC GGGCAGCAGC GGATGATCCT CCAGTATGGT
33961 AGCGCGGGTT TCTGTCTCAA AAGGAGGTAG ACGATCCCTA CTGTACGGAG TGCGCCGAGA
34021 CAACCGAGAT CGTGTGGTGC GTAGTGTCTAT GCCAAATGGA ACGCCGGACG TAGTCATATT
34081 TCCTGAAGCA AAACCAGGTG CGGGCGTGAC AAACAGATCT GCGTCTCCGG TCTCGCCGCT
34141 TAGATCGCTG TGTGTAGTAG TTGTAGTAT TCCACTCTCT CAAAGCATCC AGGCGCCCCC
34201 TGGCTTCGGG TTCTATGTAA ACTCCTTCAT GCGCCGCTGC CCTGATAACA TCCACCACCG
34261 CAGAATAAGC CACACCCAGC CAACCTACAC ATTCTGTTCTG CGAGTCACAC ACGGGAGGAG
34321 CGGGAAGAGC TGAAGAACC ATGTTTTTTT TTTTATTCCA AAAGATTATC CAAAACCTCA
34381 AAATGAAGAT CTATTAAGTG AACGCGCTCC CCTCCGGTGG CGTGGTCAAA CTCTACAGCC
34441 AAAGAACAGA TAATGGCATT TGTAAGATGT TGCACAATGG CTTCCAAAAG GCAAACGGCC
34501 CTCACGTCCA AGTGGACGTA AAGGCTAAAC CCTTCAGGGT GAATCTCCTC TATAAACATT
34561 CCAGCACCTT CAACCATGCC CAAATAATTC TCATCTCGCC ACCTTCTCAA TATATCTCTA
34621 AGCAAATCCC GAATATTAAG TCCGGCCATT GTAAAAATCT GCTCCAGAGC GCCCTCCACC
34681 TTCAGCCTCA AGCAGCGAAT CATGATTGCA AAAATTTCAGG TTCCTCACAG ACCTGTATAA
34741 GATTCAAAG CGGAACATTA ACAAAAATAC CGCGATCCCG TAGGTCCCTT CGCAGGGCCA
34801 GCTGAACATA ATCGTGCAGG TCTGCACGGA CCAGCGCGGC CACTTCCCCG CCAGGAACCT
34861 TGACAAAAGA ACCCACACTG ATTATGACAC GCATACTCGG AGCTATGCTA ACCAGCGTAG
34921 CCCCAGTGTA AGCTTTGTTG CATGGGCGGC GATATAAAAT GCAAGGTGCT GCTCAAAAAA
34981 TCAGGCAAG CCTCGCGCAA AAAAGAAAGC ACATCGTAGT CATGCTCATG CAGATAAAGG
35041 CAGGTAAGCT CCGGAACCAC CACAGAAAAA GACACCATT TTCTCTCAA CATGCTGCG
35101 GGTTTCTGCA TAAACACAAA ATAAAAATAC AAAAAACAT TTAAACATTA GAAGCCTGTC
35161 TTACAACAGG AAAACAACC CTTATAAGCA TAAGACGGAC TACGGCCATG CCGGCGTGAC
35221 CGTAAAAAAA CTGGTCACCG TGATTA AAAA GCACCACCGA CAGCTCCTCG GTCATGTCCG
35281 GAGTCATAAT GTAAGACTCG GTAAACACAT CAGGTTGATT CATCGGTCAG TGCTAAAAAG
35341 CGACCGAAAT AGCCCGGGG AATACATACC CGCAGGCGTA GAGACAACAT TACAGCCCCC
35401 ATAGGAGGTA TAACAAAATT AATAGGAGAG AAAAAACAT AAACACCTGA AAAACCTCC
35461 TGCCTAGGCA AAATAGCACC CTCCCGCTCC AGAACAACAT ACAGCGCTTC ACAGCGGCAG
35521 CCTAACAGTC AGCCTTACCA GTAAAAAGA AAACCTATTA AAAAAACACC ACTCGACAG
35581 GCACCAGCTC AATCAGTCAC AGTGTA AAAA AGGGCCAAGT GCAGAGCGAG TATATATAGG
35641 ACTAAAAAAT GACGTAACGG TTAAAGTCCA CAAAAACAC CCAGAAAACC GCACGCGAAC
35701 CTACGCCCAG AAACGAAAGC CAAAAACCC ACAACTTCCT CAAATCGTCA CTTCCGTTTT
35761 CCCACGTTAC GTAACCTCCC ATTTTAAGAA AACTACAATT CCCAACACAT ACAAGTTACT
35821 CCGCCCTAAA ACCTACGTCA CCGCCCGCT TCCCACGCCC CGCGCCACGT CACAAACTCC
35881 ACCCCCTCAT TATCATATTG GCTCAATCC AAAATAAGGT ATATTATTGA TGATG

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FIGURE 21  
(SHEET 11)

LOCUS KD1 33592 bp DNA SYN 28-APR-1999  
 DEFINITION KD1  
 ACCESSION KD1  
 KEYWORDS  
 SOURCE Unknown.  
 ORGANISM Unknown  
 Unclassified.  
 REFERENCE 1 (bases 1 to 33592)  
 AUTHORS Self  
 JOURNAL Unpublished.  
 FEATURES  
 CDS  
 Location/Qualifiers  
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 /gene="KD1"  
 /product="KD1"  
 BASE COUNT 7744 a 9470 c 9285 g 7093 t  
 ORIGIN  
 1 CATCATCAAT AATATACCTT ATTTTGGATT GAAGCCAATA TGATAATGAG GGGGTGGAGT  
 61 TTGTGACGTG GCGCGGGGCG TGGGAACGGG GCGGGTGACG TAGTAGTGTG GCGGAAGTGT  
 121 GATGTTGCAA GTGTGGCGGA ACACATGTAA GCGACGGATG TGGCAAAAGT GACGTTTTTG  
 181 GTGTGCGCGG GTGTACACAG GAAGTGACAA TTTTCGCGCG GTTTTAGGCG GATGTTGTAG  
 241 TAAATTTGGG CGTAACCGAG TAAGATTTGG CCATTTTCGC GGGAAACTG AATAAGAGGA  
 301 AGTGAAATCT GAATAATTTT GTGTTACTCA TAGCGCGTAA TATTTGTCTA GGGCCGCGGG  
 361 GACTTTGACC GTTTACGTGG AGACTCGCCC AGGTGTTTTT CTCAGGTGTT TTCGCGTTC  
 421 CGGGTCAAAG TTGGCGTTTT ATTATTATAG TCAGCTGACG TGTAGTGAT TTATACCCGG  
 481 TGAGTTCCTC AAGAGGCCAC TCTTGAGTGC CAGCGAGTAG AGTTTTCTCC TCCGAGCCGC  
 541 TCCGACACCG GGACTGAAAA TGAGACATGA GGTACTGGCT GATAATCTTC CACCTCCTAG  
 601 CCATTTTGAA CCACCTACCC TTCACGAAT GTATGATTTA GACGTGACGG CCCCCGAAGA  
 661 TCCCAACGAG GAGGCGGTTT CGCAGATTTT TCCCGACTCT GTAATGTTGG CGGTGCAGGA  
 721 AGGGATGAC TTACTCACTT TTCCGCGGCG GCCCGGTTCT CCGGAGCCGC CTCACCTTTC  
 781 CCGGCAGCCC GAGCAGCCGG AGCAGAGAGC CTTGGGTCCG GTTTGCCACG AGGCTGCGCT  
 841 TCCACCCAGT GACGACGAGG ATGAAGAGGG TGAGGAGTTT GTGTTAGATT ATGTGGAGCA  
 901 CCCCAGGCAC GGTTCAGGT CTGTGCATTA TCACCGGAGG AATACGGGGG ACCCAGATAT  
 961 TATGTGTTTC CTTTGCTATA TGAGGACCTG TGGCATGTTT GTCTACAGTA AGTGAAATTT  
 1021 ATGGGCAGTG GGTGATAGAG TGGTGGGTTT GGTGTGGTAA TTTTTTTTTT AATTTTACA  
 1081 GTTTTGTGGT TTAAAGAATT TTGTATTGTG ATTTTTTTAA AAGGTCTGT GTCTGAACCT  
 1141 GAGCCTGAGC CCGAGCCAGA ACCGGAGCCT GCAAGACCTA CCCGCCGTCC TAAATGGCG  
 1201 CCTGCTATCC TGAGACGCCC GACATCACCT GTGTCTAGAG AATGCAATAG TAGTACGGAT  
 1261 AGCTGTGACT CCGGTCCCTC TAACACACCT CCTGAGATAC ACCCGGTGGT CCCGCTGTGC  
 1321 CCCATTAAAC CAGTTGCCGT GAGAGTTGGT GGGCGTCGCC AGGCTGTGGA ATGTATCGAG  
 1381 GACTTGCTTA ACGAGCCTGG GCAACCTTGT GACTTGAGCT GTAAACGCCC CAGGCCATAA  
 1441 GGTGTAAACC TGTGATTGCG TGTGTGGTTA ACGCCTTTGT TTGCTGAATG AGTTGATGTA  
 1501 AGTTTAATAA AGGGTGAGAT AATGTTTAA TGCATGGCG TGTAAATGG GCGGGGCTT  
 1561 AAAGGGTATA TAATGCGCCG TGGGCTAATC TTGGTTACAT CTGACCTCAT GGAGGCTTGG  
 1621 GAGTGTGTTG AAGATTTTTC TGCTGTGCGT AACTTGCTGG AACAGAGCTC TAACAGTACC  
 1681 TCTTGGTTTT GGAGGTTTCT GTGGGGCTCA TCCCAGGCAA AGTTAGTCTG CAGAATTAAG  
 1741 GAGGATTACA AGTGGGAATT TGAAGAGCTT TTGAAATCCT GTGGTGAGCT GTTTGATTCT  
 1801 TTGAATCTGG GTCACCAGGC GCTTTTCCAA GAGAAGGTCA TCAAGACTTT GGATTTTTC  
 1861 ACACCGGGGC GCGCTGCGGC TGCTGTGCT TTTTGTAGTT TTATAAAGGA TAAATGGAGC  
 1921 GAAGAAACCC ATCTGAGCGG GGGGTACCTG CTGGATTTTC TGGCCATGCA TCTGTGGAGA  
 1981 GCGGTTGTGA GACACAAGAA TCGCCTGCTA CTGTTGTCTT CCGTCCGCCC GCGGATAATA  
 2041 CCGACGGAGG AGCAGCAGCA GCAGCAGGAG GAAGCCAGGC GCGGCGGCA GGAGCAGAGC  
 2101 CCATGGAACC CGAGAGCCGG CCTGGACCTT CCGGAATGAA TGTGTACAG GTGGCTGAAC  
 2161 TGTATCCAGA ACTGAGACGC ATTTTGACAA TTACAGAGGA TGGGCAGGGG CTAAGGGGG  
 2221 TAAAGAGGGA GCGGGGGGCT TGTGAGGCTA CAGAGGAGGC TAGGAATCTA GCTTTTAGCT  
 2281 TAATGACCAG ACACCGTCTT GAGTGTATTA CTTTCAACA GATCAAGGAT AATTGCGCTA  
 2341 ATGAGCTTGA TCTGCTGGCG CAGAAGTATT CCATAGAGCA GCTGACCACT TACTGGCTGC  
 2401 AGCCAGGGGA TGATTTTGAG GAGGCTATTA GGTATATGCA AAAGGTGGCA CTAGGCCAG

kd1

FIGURE 22  
(SHEET 1)

34/66

2461 ATTGCAAGTA CAAGATCAGC AAACCTGTAA ATATCAGGAA TTGTTGCTAC ATTTCTGGGA  
2521 ACGGGGCCGA GGTGGAGATA GATACGGAGG ATAGGGTGGC CTTTAGATGT AGCATGATAA  
2581 ATATGTGGCC GGGGGTGCTT GGCATGGACG GGGTGCTTAT TATGAATGTA AGGTTTACTG  
2641 GCCCCAATTT TAGCGGTACG GTTTTCCTGG CCAATACCAA CCTTATCCTA CACGGTGTAA  
2701 GCTTCTATGG GTTTAACAAT ACCTGTGTGG AAGCCTGGAC CGATGTAAGG GTTCGGGGCT  
2761 GTGCTTTTTA CTGCTGCTGG AAGGGGGTGG TGTGTCGCCC CAAAAGCAGG GCTTCAATTA  
2821 AGAAATGCCT CTTTGAAAGG TGTACCTTGG GTATCCTGTC TGAGGGTAAC TCCAGGGTGC  
2881 GCCACAATGT GGCCTCCGAC TGTGGTTGCT TCATGCTAGT GAAAAGCGTG GCTGTGATTA  
2941 AGCATAACAT GGTATGTGGC AACTGCGAGG ACAGGGCCTC TCAGATGCTG ACCTGCTCGG  
3001 ACGGCAACTG TCACCTGCTG AAGACCATT CAGTAGCCAG CCACTCTCGC AAGGCCTGGC  
3061 CAGTGTTTGA GCATAACATA CTGACCCGCT GTTCCTTGCA TTTGGGTAAC AGGAGGGGGG  
3121 TGTTCCTACC TTACCAATGC AATTTGAGTC ACACCTAAGT ATTGCTTGAG CCGAGAGCA  
3181 TGTCCAAGGT GAACCTGAAC GGGGTGTTTG ACATGACCAT GAAGATCTGG AAGGTGCTGA  
3241 GGTACGATGA GACCCGCACC AGGTGCAGAC CCTGCGAGTG TGGCGGTAAA CATATTAGGA  
3301 ACCAGCCTGT GATGCTGGAT GTGACCGAGG AGCTGAGGCC CGATCACTTG GTGCTGGCCT  
3361 GCACCCGCGC TGAGTTTGGC TCTAGCGATG AAGATACAGA TTGAGGTACT GAAATGTGTG  
3421 GCGTGCGCTT AAGGGTGGGA AAGAATATAT AAGGTGGGGG TCTTATGTAG TTTTGTATCT  
3481 GTTTTGACG AGCCGCCGCC GCCATGAGCA CCAACTCGTT TGATGGAAGC ATTGTGAGCT  
3541 CATATTGAC AACCGCATG CCCCATGGG CCGGGGTGCG TCAGAATGTG ATGGGCTCCA  
3601 GCATTGATGG TCGCCCCGTC CTGCCCCGAA ACTCTACTAC CTTGACCTAC GAGACCGTGT  
3661 CTGGAACGCC GTTGGAGACT GCAGCCTCCG CCGCCGCTTC AGCCGCTGCA GCCACCGCCC  
3721 GCGGGATTGT GACTGACTTT GCTTTCCTGA GCCCGCTTGC AAGCAGTGCA GCTTCCCGTT  
3781 CATCCGCCCG CGATGACAAG TTGACGGCTC TTTTGGCACA ATTGGATTCT TTGACCCGGG  
3841 AACTTAATGT CGTTTCTCAG CAGCTGTGG ATCTGCGCCA GCAGGTTTCT GCCCTGAAGG  
3901 CTCTCTCCCC TCCCAATGCG GTTTAAACA TAAATAAAAA ACCAGACTCT GTTTGGATT  
3961 GGATCAAGCA AGTGTCTTGC TGTCTTTATT TAGGGGTTTT GCGCGCGCGG TAGGCCCGGG  
4021 ACCAGCGGTC TCGGTCTGTG AGGGTCTGT GTATTTTTTC CAGGACGTGG TAAAGGTGAC  
4081 TCTGGATGTT CAGATACATG GGCATAAGCC CGTCTCTGGG GTGGAGGTAG CACCACTGCA  
4141 GAGCTTCATG CTGCGGGGTG GTGTTGTAGA TGATCCAGTC GTAGCAGGAG CGCTGGGCGT  
4201 GGTGCCTAAA AATGTCTTTC AGTAGCAAGC TGATTGCCAG GGGCAGGCCC TTGGTGTAA  
4261 TGTTTACAAA GCGGTTAAGC TGGGATGGGT GCATACGTGG GGATATGAGA TGCATCTTGG  
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18601 ATCGTGGGTC TGGGGGTGCA ATCCCTGAAG CGCCGACGAT GCTTCTGAAT AGCTAACGTG
18661 TCGTATGTGT GTCATGTATG CGTCAATGTC GCGCCAGAG GAGCTGCTGA GCGCGCGCGC
18721 GCCCGCTTTC CAAGATGGCT ACCCTTCCA TGATGCCGCA GTGGTCTTAC ATGCACATCT
18781 CGGGCCAGGA CGCCTCGGAG TACCTGAGCC CCGGGTGGT GCAGTTTGCC CGCGCCACCG
18841 AGACGTACTT CAGCCTGAAT AACAAGTTTA GAAACCCAC GGTGGCGCCT ACGCACGACG
18901 TGACCACAGA CCGGTCCAG CGTTTGACGC TGCGGTTTCT CCCTGTGGAC CGTGAGGATA
18961 CTGCGTACTC GTACAAGCG CGGTTACCC TAGCTGTGGG TGATAACCGT GTGCTGGACA
19021 TGGCTTCCAC GTACTTTGAC ATCCGCGGCG TGCTGGACAG GGGCCCTACT TTTAAGCCCT
19081 ACTCTGGCAC TGCCCTACAAC GCCCTGGCTC CCAAGGTTGC CCAAAATCCT TGCGAATGGG
19141 ATGAAGCTGC TACTGCTCTT GAAATAAACC TAGAAGAAGA GGACGATGAC AACGAAGACG
19201 AAGTAGACGA GCAAGCTGAG CAGCAAAAAA CTCACGTATT TGGGCAGGCG CTTTATCTG
19261 GTATATAATAT TACAAAGGAG GGTATTCAAA TAGGTGTCGA AGGTCAAACA CCTAAATATG
19321 CCGATAAAAC ATTTCAACCT GAACCTCAA TAGGAGAATC TCAGTGGTAC GAAACTGAAA
19381 TTAATCATGC AGCTGGGAGA GTCCTTAAAA AGACTACCCC AATGAAACCA TGTACCGTT
19441 CATATGCAAA ACCACAAAT GAAATGGAG GGCAAGGCAT TCTGTAAAG CAACAAAATG
19501 GAAAGCTAGA AAGTCAAGTG GAAATGCAAT TTTTCTCAAC TACTGAGGCG ACCGCAGGCA

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19561 ATGGTGATAA CTTGACTCCT AAAAGTGGTAT TGTACAGTGA AGATGTAGAT ATAGAAACCC
19621 CAGACACTCA TATTTCTTAC ATGCCCACTA TTAAGGAAGG TAACTCACGA GAACTAATGG
19681 GCCAACAAATC TATGCCCAAC AGGCCTAATT ACATTGCTTT TAGGGACAAT TTTATTGGTC
19741 TAATGTATTA CAACAGCACG GGTAAATATGG GTGTTCTGGC GGGCCAAGCA TCGCAGTTGA
19801 ATGCTGTTGT AGATTTGCAA GACAGAAACA CAGAGCTTTC ATACCAGCTT TTGCTTGATT
19861 CCATTGGTGA TAGAACCAGG TACTTTTCTA TGTGGAATCA GGCTGTTGAC AGCTATGATC
19921 CAGATGTTAG AATTATTGAA AATCATGGAA CTGAAGATGA ACTTCCAAAT TACTGCTTTC
19981 CACTGGGAGG TGTGATTAAT ACAGAGACTC TTACCAAGGT AAAACCTAAA ACAGGTCAGG
20041 AAAATGGATG GGA AAAAGAT GCTACAGAAT TTTTCAGATA AAATGAAATA AGAGTTGGAA
20101 ATAATTTTGC TAGGAAATC AATCTAAATG CCAACCTGTG GAGAAATTTT CTGTACTCCA
20161 ACATAGCGCT GTATTTGCCG GACAAGCTAA AGTACAGTCC TTCCAACGTA AAAATTTCTG
20221 ATAACCCAAA CACCTACGAC TACATGAACA AGCGAGTGGT GGCTCCCGGG TTAGTGGACT
20281 GCTACATTAA CCTTGGAGCA CGCTGGTCCC TTGACTATAT GGACAACGTC AACCATTITA
20341 ACCACCACCG CAATGCTGGC CTGCGCTACC GCTCAATGTT GCTGGGCAAT GGTCGCTATG
20401 TGCCCTTCCA CATCCAGGTG CCTCAGAAGT TCTTTGCCAT TAAAAACCTC CTTCTCCTGC
20461 CGGGCTCATA CACCTACGAG TGGAACTTCA GGAAGGATGT TAACATGGTT CTGCAGAGCT
20521 CCTAGGAAA TGACCTAAGG GTTGACGGAG CCAGCATTAA GTTTGATAGC ATTTGCCTTT
20581 ACGCCACCTT CTTCCCATG GCCACAACA CCGCTCCAC GCTTGAGGCC ATGCTTAGAA
20641 ACGACACCAA CGACCACTCC TTTAAGACT ATCTCTCCGC CGCCAACATG CTCTACCCTA
20701 TACCCGCCAA CGCTACCAAC GTGCCATAT CCATCCCCTC CCGCAACTGG GCGGCTTTCC
20761 GCGGCTGGGC CTTACGCGC CTTAAGACTA AGGAAACCCC ATCACTGGGC TCGGGCTACG
20821 ACCCTTATTA CACCTACTCT GGCTCTATAC CCTACCTAGA TGAACCTTT TACCTCAAC
20881 AACCTTTTAA GAAGGTGGCC ATTACCTTTG ACTCTTCTGT CAGCTGGCCT GGCAATGACC
20941 GCCTGCTTAC CCCCACGAG TTTGAAATTA AGCGCTCAGT TGACGGGGAG GGTTACAACG
21001 TTGCCCAGTG TAACATGACC AAAGACTGGT TCCTGGTACA AATGCTAGCT AACTACAACA
21061 TTGGCTACCA GGGCTTCTAT ATCCAGAGA GCTACAAGGA CCGCATGTAC TCCTTCTTTA
21121 GAAACTTCCA GCCCATGAGC CGTCAGGTGG TGGATGATAC TAAATACAAG GACTACCAAC
21181 AGGTGGGCAT CCTACACCAA CACAACAAC CTGGATTTGT TGGCTACCTT GCGCCACCA
21241 TGCGCGAAGG ACAGGCCTAC CCTGCTAAGT TCCCCTATCC GCTTATAGGC AAGACCGCAG
21301 TTGACAGCAT TACCCAGAAA AAGTTTCTTT GCGATCGCAC CCTTTGGGCG ATCCCATCTT
21361 CCAGTAACCT TATGTCCATG GCGCACTCA CAGACCTGGG CCAAAACCTT CTCTACGCCA
21421 ACTCCGCCCA CGCGTAGAC ATGACTTTTG AGGTGGATCC CATGGACGAG CCCACCTTC
21481 TTTATGTTTT GTTTGAAGTC TTTGACGTGG TCCGTGTGCA CCGGCCGCAC CGCGGCGTCA
21541 TCGAAACCGT GTACCTGCGC ACGCCCTTCT CGGCCGGCAA CGCCACAACA TAAAGAAGCA
21601 AGCAACATCA ACAACAGCTG CCGCATGGG CTCCAGTGAG CAGGAAGTGA AAGCCATTGT
21661 CAAAGATCTT GGTGTGGGC CATATTTTTT GGGCACCTAT GACAAGCGCT TTCCAGGCTT
21721 TGTTTCTCCA CACAAGCTCG CCTGCGCCAT AGTCAATACG GCCGGTCGCG AGACTGGGGG
21781 CGTACACTGG ATGGCCTTTG CCTGGAACCC GCACTCAAAA ACATGCTACC TCTTTGAGCC
21841 CTTTGGCTTT TCTGACCAGC GACTCAAGCA GGTTTACCAG TTTGAGTACG AGTCACTCCT
21901 GCGCGTAGC GCCATTGCTT CTTCCCCCGA CCGCTGTATA ACGCTGAAA AGTCCACCCA
21961 AAGCGTACAG GGGCCCACT GGGCGCCTG TGGACTATTC TGCTGCATGT TTCTCCACGC
22021 CTTTGGCCAA TGGCCCCAAA CTCCCATGGA TCACAACCCC ACCATGAACC TTATTACCGG
22081 GGTACCCAAC TCCATGCTCA ACAGTCCCCA GGTACAGCCC ACCCTGCGTC GCAACCAGGA
22141 ACAGCTCTAC AGCTTCCTGG AGCGCCACTC GCCCTACTTC CGCAGCCACA GTGCGCAGAT
22201 TAGGAGCGCC ACTTCTTTTT GTCACCTGAA AAACATGTAA AAATAATGTA CTAGAGACAC
22261 TTTCAATAAA GGCAATGCT TTTATTGTGA CACTCTCGGG TGATTATTTA CCCCCACCCT
22321 TGCCGTCTGC GCCGTTTAAA AATCAAAGGG GTTCTGCCGC GCATCGCTAT GCGCCACTGG
22381 CAGGGACACG TTGCGATACT GGTGTTTAGT GCTCCACTTA AACTCAGGCA CAACCATCCG
22441 CCGCAGCTCG GTGAAGTTTT CACTCCACAG GCTGCGCACC ATCACCAACG CGTTTAGCAG
22501 GTCGGGCGCC GATATCTTGA AGTCGCAGTT GGGGCTCCG CCCTGCGCGC GCGAGTTGCG
22561 ATACACAGGG TTGCAGCACT GGAACACTAT CAGCGCCGGG TGGTGACGCG TGGCCAGCAC
22621 GCTCTTGTG GAGATCAGAT CCGCTCCAG GTCTCCGCG TTGCTCAGGG CGAACGGAGT
22681 CAACTTTGGT AGCTGCCTTC CCAAAAAGGG CGCGTGCCCA GGCTTTGAGT TGCACTCGCA
22741 CCGTAGTGCC ATCAAAAGGT GACCGTGCCC GGTCTGGGCG TTAGGATACA GCGCCTGCAT
22801 AAAAGCCTTG ATCTGCTTAA AAGCCACCTG AGCCTTTGCG CCTTCAGAGA AGAACATGCC
22861 GCAAGACTTG CCGGAAAAC GATTGGCCGG ACAGGCCGCG TCGTGACGCG AGCACCTTGC
22921 GTCGGTGTG GAGATCTGCA CCACATTTG GCCCCACCGG TTCTTCACGA TCTTGGCCTT

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22981 GCTAGACTGC TCCTTCAGCG CGCGCTGCCC GTTTTCGCTC GTCACATCCA TTTCAATCAC
23041 GTGCTCCTTA TTTATCATAA TGCTTCGGTG TAGACACTTA AGCTCGCCTT CGATCTCAGC
23101 GCAGCGGTGC AGCCACAACG CGCAGCCCGT GGGCTCGTGA TGCTTGTAAG TCACCTCTGC
23161 AAACGACTGC AGGTACGCCT GCAGGAATCG CCCCATCATC GTCACAAAGG TCTTGTTGCT
23221 GGTGAAGGTC AGCTGCAACC CGCGGTGCTC CTCGTTCAGC CAGGTCTTGC ATACGGCCGC
23281 CAGAGCTTCC ACTTGGTCAG GCAGTAGTIT GAAGTTCGCC TTTAGATCGT TATCCACGTG
23341 GTACTTGTCC ATCAGCGCGC GCGCAGCCTC CATGCCCTTC TCCCACGCAG ACACGATCGG
23401 CACACTCAGC GGGTTCATCA CCGTAATTTC ACTTTCGGCT TCGTGGGCT CTTCTCTTTC
23461 CTCTTGCGTC CGCATACCAC GCGCCACTGG GTCGTCTTCA TTCAGCCGCC GCACTGTGCG
23521 CTTACCTCCT TTGCCATGCT TGATTAGCAC CGGTGGGTTG CTGAAACCCA CCATTGTAG
23581 CGCCACATCT TCTCTTCTT CCTCGCTGTC CACGATTACC TCTGGTGATG GCGGGCGCTC
23641 GGGCTTGGGA GAAGGGCGCT TCTTTTCTT CTTGGGCGCA ATGGCCAAAT CCGCCGCGA
23701 GGTCTGATGC CGCGGGCTGG GTGTGCGCGG CACCAGCGCG TCTTGTGATG AGTCTTCTC
23761 GTCCTCGGAC TCGATACGCC GCCTCATCCG CTTTTTTGGG GGCGCCCGGG GAGGCGGGG
23821 CGACGGGGAC GGGGACGACA CGTCTCCAT GGTGGGGGA CGTCGCGCCG CACCGCGTCC
23881 GCGCTCGGGG GTGGTTTTCGC GCTGCTCTC TTCCCGACTG GCCATTTCCT TCTCTATAG
23941 GCAGAAAAG ATCATGGAGT CAGTCGAGAA GAAGGACAGC CTAACCGCCC CCTCTGAGTT
24001 CGCCACCACC GCCTCCACCG ATGCCGCCAA CGCGCCTACC ACCTTCCCCG TCGAGGCACC
24061 CCGCTTGGAG GAGGAGGAAG TGATTATCGA GCAGGACCCA GGTTTTGTAA GCGAAGACGA
24121 CGAGGACCGC TCAGTACCAA CAGAGGATAA AAAGCAAGAC CAGGACAACG CAGAGGCAAA
24181 CGAGGAACAA GTCGGGCGGG GGGACGAAAG GCATGGCGAC TACCTAGATG TGGGAGACGA
24241 CGTGCTGTTG AAGCATCTGC AGCGCCAGTG CGCCATTATC TGCGACCGGT TGCAAGAGCG
24301 CAGCGATGTG CCCCTCGCCA TAGCGGATGT CAGCCTTGCC TACGAACGCC ACCTATTCTC
24361 ACCGCGCGTA CCCCCCAAAC GCCAAGAAA CGGCACATGC GAGCCCAACC CGCGCTCAA
24421 CTTCTACCCC GTATTTGCCG TGCCAGAGGT GCTTGCCACC TATCACATCT TTTTCAAAA
24481 CTGCAAGATA CCCCTATCCT GCCGTGCCAA CCGCAGCCGA GCGGACAAGC AGCTGGCCTT
24541 GCGGCAGGGC GCTGTCTATC CTGATATCGC CTCGCTCAAC GAAGTGCCAA AAATCTTTGA
24601 GGGTCTTGGG CGCGACGAGA AGCGCGCGGC AAACGCTCTG CAACAGGAAA ACAGCGAAAA
24661 TGAAAGTCAC TCTGGAGTGT TGGTGGAACT CGAGGGTGAC AACGCGCGCC TAGCCGTACT
24721 AAAACGCAGC ATCGAGGTCA CCCACTTTTG CTACCCGCGA CTTAACCTAC CCCCAGGT
24781 CATGAGCACA GTCATGAGTG AGCTGATCGT GCGCCGTGCG CAGCCCCTGG AGAGGGATGC
24841 AAATTGCAA GAACAAACAG AGGAGGGCCT ACCCGCAGTT GCGACGAGC AGCTAGCGCG
24901 CTGGCTTCAA ACGCGCGAGC CTGCCGACTT GGAGGAGCGA CGCAAACATA TGATGGCCGC
24961 AGTGCTCGTT ACCGTGGAGC TTGAGTGCAAT GCAGCGGTTT TTTGCTGACC CGGAGATGCA
25021 GCGCAAGCTA GAGGAAACAT TGCACTACAC CTTTCGACAG GGCTACGTAC GCCAGGCCTG
25081 CAAGATCTCC AACGTGGAGC TCTGCAACCT GGTCTCCTAC CTTGGAATTT TGCACGAAAA
25141 CCGCCTTGGG CAAAACGTGC TTCAATCCAC GCTCAAGGSC GAGGCGCGCC GCGACTACGT
25201 CCGCGACTGC GTTACTTAT TTCTATGCTA CACCTGGCAG ACGGCCATGG GCGTTTGGCA
25261 GCAGTGCTTG GAGGAGTGCA ACCTCAAGGA GCTGCAGAAA CTGCTAAAGC AAAACTTGAA
25321 GGACCTATGG ACGGCCTTCA ACGAGCGCTC CGTGGCCGCG CACCTGGCGG ACATCATTTT
25381 CCCCAGACGC CTGCTTAAAA CCCTGCAACA GGGTCTGCCA GACTTCACCA GTCAAAGCAT
25441 GTTGACAGAC TTTAGGAACT TTATCCTAGA GCGCTCAGGA ATCTTGCCCG CCACCTGCTG
25501 TGCACTTCTT AGCGACTTTG TGCCCAATTA GTACCGCGAA TGCCCTCCGC CGCTTTGGGG
25561 CCACTGCTAC CTTCTGCAGC TAGCCAATA CTTGCTTAC CACTCTGACA TAATGGAAGA
25621 CGTGAGCGGT GACGGTCTAC TGGAGTGTC CTGTGCTGTC AACCTATGCA CCGCGCACCG
25681 CTCCTGGTT TGCAATTGCG AGCTGCTTAA CGAAAGTCAA ATTATCGGTA CCTTTGAGCT
25741 GCAGGGTCCC TCGCTGACG AAAAGTCCGC GGCTCCGGGG TTGAAACTCA CTCCGGGGCT
25801 GTGGACGTG GCTTACCTTC GCAAATTTGT ACCTGAGGAC TACCACGCC ACAGATTAG
25861 GTTCTACGAA GACCAATCCC GCCCGCAAAA TGCGGAGCTT ACCGCTGCG TCATTACCCA
25921 GGGCCACATT CTTGGCCAAT TGCAAGCCAT CAACAAAGCC CGCCAAGAGT TTCTGCTACG
25981 AAAGGGACGG GGGGTTTACT TGGACCCCA GTCCGGCGAG GAGCTCAACC CAATCCCCC
26041 GCCGCGCAG CCCTATCAGC AGCAGCGCG GGCCTTGCT TCCAGGATG GCACCCAAA
26101 AGAAGCTGCA GCTGCCGCCG CACCCACGG ACGAGGAGGA ATACTGGGAC AGTCAGGCAG
26161 AGGAGGTTTT GGACGAGGAG GAGGAGGACA TGATGGAAGA CTGGGAGAGC CTAGACGAGG
26221 AAGCTTCCGA GTTCGAAGAG GTGTCAGAGG AAACACCGTC ACCCTCGGTC GCATTCCCTT
26281 CGCCGCGGCC CCAGAAATCG GCAACCGGTT CCAGCATGGC TACAACCTCC GCTCCTCAGG
26341 CGCCGCGGCC ACTGCCCGTT CGCCGACCCA ACCGTAGATG GGACACCACT GGAACCAAGG

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26401 CCGGTAAGTC CAAGCAGCCG CCGCCGTTAG CCCAAGAGCA ACAACAGCGC CAAGGCTACC
26461 GCTCATGGCG CGGGCACAAG AACGCCATAG TTGCTTGCTT GCAAGACTGT GGGGGCAACA
26521 TCTCCTTCGC CCGCCGCTTT CTTCCTTACC ATCACGGCGT GGCCTTCCCC CGTAACATCC
26581 TGCATTACTA CCGTCATCTC TACAGCCCAT ACTGCACCGG CGGCAGCGGC AGCGGCAGCA
26641 ACAGCAGCGG CCACACAGAA GCAAAGGCGA CCGGATAGCA AGACTCTGAC AAAGCCCAAG
26701 AAATCCACAG CGGCGGCAGC AGCAGGAGGA GGAGCGCTGC GTCTGGCGCC CAACGAACCC
26761 GTATCGACCC GCGAGCTTAG AAACAGGATT TTTCCCACTC TGTATGCTAT ATTTCAACAG
26821 AGCAGGGGCC AAGAACAAGA GCTGAAAATA AAAACAGGT CTCTGCGATC CCTCACCCTG
26881 AGCTGCCTGT ATCACAAGAG CGAAGATCAG CTTGCGCGCA CGCTGGAAGA CGCGGAGGCT
26941 CTCTTCAGTA AATACTGCGC GCTGACTCTT AAGGACTAGT TTCGCGCCCT TTCTCAAATT
27001 TAAGCGCGAA AACTACGTCA TCTCCAGCGG CCACACCCGG CGCCAGCACC TGTCGTGAGC
27061 GCCATTATGA GCAAGGAAAT TCCCACGCCC TACATGTGGA GTTACCAGCC ACAAATGGGA
27121 CTTGCGGCTG GAGCTGCCCA AGACTACTCA ACCCGAATAA ACTACATGAG CGCGGGACCC
27181 CACATGATAT CCCGGGTCAA CGGAATCCGC GCCACCGAA ACCGAATTCT CTTGGAACAG
27241 GCGGCTATTA CCACCACACC TCGTAATAAC CTTAATCCCC GTAGTTGGCG CGCTGCCCTG
27301 GTGTACCAGG AAAGTCCCGC TCCCACACT GTGGTACTTC CCAGAGACGC CCAGGCCGAA
27361 GTTCAGATGA CTAATCAGG GCGCGAGCTT GCGGGCGGCT TTCGTACAG GGTGCGGTG
27421 CCGGGCAGG GTATAACTCA CCTGACAATC AGAGGGGAG GTATTGAGT CAACGACGAG
27481 TCGGTGAGCT CCTCGCTTG TCTCCGTCCG GACGGGACAT TTCAGATCGG CGCGCGCGGC
27541 CGTCCCTCAT TCACGCCTCG TCAGGCAATC CTAATCTGTC AGACCTCGTC CTCTGAGCCG
27601 CGCTCTGGAG GCATTGGAAC TCTGCAATTT ATTGAGGAGT TTGTGCCATC GGTCTACTTT
27661 AACCCTTCT CGGGACCTCC CGGCCACTAT CCGGATCAAT TTATTCTTAA CTTTGACGCG
27721 GTAAAGGACT CGGCGGACGG CTACGACTGA TAATTAAAGT GAGAGGCAGA GCAACTGCGC
27781 CTGAAACACC TGGTCCACTG TCGCCGCCAC AAGTGCTTTG CCCGCGACTC CGGTGAGTTT
27841 TGCTACTTTG AATTGCCCGA GGATCATATC GAGGATCTTT GTTGCCATCT CTGTGCTGAG
27901 TATAATAAAT ACAGAAATTA AAATATACTG GGGCTCCTAT CGCCATCCTG TAAACGCCAC
27961 CGCTTTCACC CGCCCAAGCA AACCAAGGCG AACCTTACCT GGTACTTTTA ACATCTCTCC
28021 CTCTGTGATT TACAACAGTT TCAACCCAG CGGAGTGAGT CTACGAGAGA ACCTCTCCGA
28081 GCTCAGCTAC TCCATCAGAA AAAACACCAC CCTCCTTACC TGCCGGGAAC GTACCCTTAA
28141 TTAAAGTCA GGCTTCTTGG ATGTCAGCAT CTGACTTTGG CCAGCACCTG TCCCGCGGAT
28201 TTGTTCCAGT CCAACTACAG CGACCCACCC TAACAGAGAT GACCAACACA ACCAACGCGG
28261 CCGCCGCTAC CGGACTTACA TCTACCACAA ATACACCCCA AGTTTCTGCC TTGTGCAATA
28321 ACTGGGATAA CTGGGCATG TGGTGGTTCT CCATAGCGCT TATGTTTGTA TGCCTTATTA
28381 TTATGTGGCT CATCTGCTGC CTAAGCGCA AACGCGCCG ACCACCCATC TATAGTCCCA
28441 TCATTGTGCT ACACCCAAAC AATGATGGAA TCCATAGATT GGACGGACTG AAACACATGT
28501 TCTTTTCTCT TACAGTATGA TTAAATGAGA TTAATTAAAG AATTCTGTC CAGTTTATTC
28561 AGCAGCACCT CTTGCCCTC CTCCAGCTC TGGTATTGCA GCTTCTCCT GGCTGCAAAC
28621 TTTCTCCACA ATCTAAATGG AATGTCAGTT TCCTCCTGTT CCTGTCCATC CGCACCCACT
28681 ATCTTCATGT TGTGTCAGT GAAGCGCGCA AGACCGTCTG AAGATACCTT CAACCCCGTG
28741 TATCCATATG ACACGGAAC CGGTCTCTCA ACTGTGCTT TTCTTACTCC TCCCTTTGTA
28801 TCCCCAATG GGTTCAGA GAGTCCCCCT GGGTACTCT CTTTGCCTT ATCCGAACCT
28861 CTAGTTACCT CCAATGGCAT GCTTGCCTC AAAATGGGCA ACGGCTCTC TCTGGACGAG
28921 GCCGGCAACC TTACCTCCCA AAATGTAACC ACTGTGAGCC CACCTCTCAA AAAAACCAAG
28981 TCAACATAA ACCTGGAAT ATCTGCACCC CTCACAGTTA CCTCAGAAGC CCTAACTGTG
29041 GCTGCCGCCG CACCTCTAAT GGTGCGGGC AACACACTCA CCATGCAATC ACAGGCCCGG
29101 CTAACCGTGC ACGACTCCAA ACTTAGCATT GCCACCCAAG GACCCCTCAC AGTGTGAGAA
29161 GGAAAGCTAG CCCTGCAAC ATCAGGCCCC CTCACCACA CCGATAGCAG TACCCTTACT
29221 ATCACTGCCT CACCCCTCT AACTACTGCC ACTGGTAGCT TGGGCATTGA CTGAAAGAG
29281 CCCATTTATA CACAAAATGG AAAACTAGGA CTAAAGTACG GGGCTCCTTT GCATGTAACA
29341 GACGACCTAA ACACCTTTGAC CGTAGCACT GGTCCAGGTG TGAATATTAA TAATACTTCC
29401 TTGCAAACTA AAGTTACTGG AGCCTTGGGT TTTGATTAC AAGGCAATAT GCAACTTAAT
29461 GTAGCAGGAG GACTAAGGAT TGATTCTCAA AACAGACGCC TTATACTTGA TGTAGTTAT
29521 CCGTTTGATG CTCAAAACCA ACTAAATCTA AGACTAGGAC AGGGCCCTCT TTTATAAAC
29581 TCAGCCACCA ACTTGATAT TAACATAAC AAAGGCCCTT ACTTGTTTAC AGCTTCAAAC
29641 AATTCCAAAA AGCTTGAGGT TAACCTAAGC ACTGCCAAGG GGTGATGTT TGACGCTACA
29701 GCCATAGCCA TTAATGCAGG AGATGGGCTT GAATTGGTT CACCTAATGC ACCAAACACA
29761 AATCCCTCA AAACAAAAT TGGCCATGGC CTAGAATTG ATTCAAACAA GGCTATGGTT

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FIGURE 22  
(SHEET 9)

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29821 CCTAACTAG GAACTGGCCT TAGTTTTGAC AGCACAGGTG CCATTACAGT AGGAAACAAA  
29881 AATAATGATA AGCTAACTTT GTGGACCACA CCAGTCCAT CTCTAACTG TAGACTAAAT  
29941 GCAGAGAAAG ATGCTAACT CACTTTGGTC TTAACAAAAT GTGGCAGTCA AATACTTGCT  
30001 ACAGTTTCAG TTTTGGCTGT TAAAGGCAGT TTGGCTCCAA TATCTGGAAC AGTTCAAAGT  
30061 GCTCATCTTA TTATAAGATT TGACGAAAAT GGAGTGCTAC TAAACAATTC CTTCCTGGAC  
30121 CCAGAATATT GGAACTTTAG AAATGGAGAT CTTACTGAAG GCACAGCCTA TACAAACGCT  
30181 GTTGGATTTA TGCCTAACCT ATCAGCTTAT CCAAAATCTC ACGGTAAAAC TGCCAAAAGT  
30241 AACATTGTCA GTCAAGTTTA CTAAACGGA GACAAAATA AACCTGTAAC ACTAACCATT  
30301 AACTAAACG GTACACAGGA AACAGGAGAC ACAACTCCAA GTGCATACTC TATGTCTATT  
30361 TCATGGGACT GGTCTGGCCA CAACTACATT AATGAAATAT TTGCCACATC CTCTTACACT  
30421 TTTTCATACA TTGCCCAAGA ATAAAGAATC GTTTGTGTTA TGTTTCAACG TGTTTATTTT  
30481 TCAATTGCAG AAAATTTCAA GTCATTTTTC ATTCAAGTAG ATAGCCCCAC CACCACATAG  
30541 CTTATACAGA TCACCGTACC TTAATCAAAC TCACAGAACC CTAGTATTCA ACCTGCCACC  
30601 TCCCTCCCAA CACACAGAGT ACACAGTCCT TTCTCCCCGG CTGGCCTTAA AAAGCATCAT  
30661 ATCATGGGTA ACAGACATAT TCTTAGGTGT TATATCCAC ACGGTTTCCT GTCGAGCCAA  
30721 ACGCTCATCA GTGATATTAA TAAACTCCCC GGGCAGCTCA CTTAAGTTCA TGTCGCTGTC  
30781 CAGCTGCTGA GCCACAGGCT GCTGTCCAAC TTGCGGTTGC TTAACGGGCG GCGAAGGAGA  
30841 AGTCCACGCG TACATGGGGG TAGAGTCATA ATCGTGATC AGGATAGGGC GGTGGTGCTG  
30901 CAGCAGCGCG CGAATAAACT GCTGCCGCGG CCGCTCCGTC CTGCAGGAAT ACAACATGCG  
30961 AGTGGTCTCC TCAGCGATGA TTGCGACGCG CCGCAGCATA AGGCGCCTTG TCCTCCGGGC  
31021 ACAGCAGCGC ACCCTGATCT CACTTAAATC AGCACAGTAA CTGCAGCACA GCACCACAT  
31081 ATTGTTCAAA ATCCACAGT GCAAGGCGCT GTATCCAAAG CTCATGGCGG GGACCACAGA  
31141 ACCCAGCTGG CCATCATACC ACAAGCGCAG GTAGATTAAG TGGCGACCCC TCATAAACAC  
31201 GCTGGACATA AACATTACCT CTTTGGCATC GTTGTAAATC ACCACCTCCC GGTACCATAT  
31261 AAACCTCTGA TTAAACATGG CGCCATCCAC CACCATCCTA AACCAGCTGG CCAAAACCTG  
31321 CCGCGCGGCT ATACACTGCA GGAACCGGG ACTGGAACAA TGACAGTGGA GAGCCCAGGA  
31381 CTCGTAACCA TGGATCATCA TGCTCGTCAT GATATCAATG TTGGCACAAC ACAGGCACAC  
31441 GTGCATACAC TTCTCAGGA TTACAAGCTC CTCCCGGCTT AGAACCATAT CCCAGGGAAC  
31501 AACCATTCC TGAATCAGCG TAAATCCCACT ACTGCAGGGA AGACCTCGCA CGTAATCACC  
31561 GTTGTGCATT GTCAAAGTGT TACATTGCGG CAGCAGCGGA TGATCTCCA GTATGGTAGC  
31621 GCGGTTTTCT GTCTCAAAG GAGGTAGACG ATCCCTACTG TACGGAGTGC GCGGAGACAA  
31681 CCGAGATCGT GTTGGTCGTA GTGTCATGCC AAATGGAACG CCGGACGTAG TCATATTTCC  
31741 TGAAGCAAAA CCAGGTGCGG GCGTGACAAA CAGATCTGCG TCTCCGGTCT CGCCGCTTAG  
31801 ATCGCTCTGT GTAGTAGTTG TAGTATATCC ACTCTCTCAA AGCATCCAGG CGCCCCCTGG  
31861 CTTGCGGTTT TATGTAACT CTTTCATGCG CCGCTGCCCT GATAACATCC ACCACCGCAG  
31921 AATATCCGAA TATTAAGTCC CCTACACATT CGTCTGCGA GTCACACACG GGAGGAGCGG  
31981 GAAGAGCTGG AAGAACCATG TTTTTTTTTT TATTCAAAA GATTATCCAA AACCTCAAAA  
32041 TGAAGATCTA TTAAGTGAAC GCGCTCCCCT CCGGTGGCGT GGTCAAATC TACAGCCAAA  
32101 GAACAGATAA TGGCATTGTG AAGATGTTGC ACAATGGCTT CCAAAAGGCA AACGGCCCTC  
32161 ACGTCCAAGT GGACGTAAAG GCTAAACCTT TCAGGGTGAA TCTCCTCTAT AAACATTCCA  
32221 GCACCTTCAA CCATGCCCAA ATAATTCTCA TCTCGCCACC TTCTCAATAT ATCTTAAGC  
32281 AAATCCCGAA TATTAAGTCC GGCCATTGTA AAAATCTGCT CCAGAGCGCC CTCCACCTTC  
32341 AGCCTCAAGC AGCGAATCAT GATTGCAAAA ATTCAAGTTC CTCACAGACC TGTATAAGAT  
32401 TCAAAGCGG AACATTACA AAAATACCGC GATCCGCTAG GTCCCTTCGC AGGGCCAGCT  
32461 GAACATAATC GTGCAGGTCT GCACGGACCA GCGCGGCCAC TTCCCCGCCA GGAACCTTGA  
32521 CAAAAGAACC CACACTGATT ATGACACGCA TACTCGGAGC TATGCTAACC AGCGTAGCCC  
32581 CGATGTAAGC TTTGTTGCAT GGGCGGCGAT ATAAAATGCA AGGTGCTGCT CAAAAATCA  
32641 GGCAAAGCCT CGCGCAAAA AGAAAGCACA TCGTAGTCAT GCTCATGCAG ATAAAGGCAG  
32701 GTAAGCTCCG GAACCACCAC AGAAAAAGAC ACCATTTTTC TCTCAAACAT GTCTGCGGGT  
32761 TTCTGCATAA ACACAAAATA AAATAACAAA AAAACATTTA AACATTAGAA GCCTGTCTTA  
32821 CAACAGGAAA AACAACCTT ATAAGCATAA GACGGACTAC GGCCATGCCG GCGTGACCGT  
32881 AAAAAAAGTGT GTACCCGTGA TTA AAAAGCA CCACCGACAG CTCTCGGTG ATGTCCGGAG  
32941 TCATAATGTA AGACTCGGTA AACACATCAG GTTGATTTCAT CGGTCAAGTGC TAAAAAGCGA  
33001 CCGAAATAGC CCGGGGGAAT ACATACCCGC AGGCGTAGAG ACAACATTAC AGCCCCATA  
33061 GGAGGTATAA CAAAATTAAT AGGAGAGAAA AACACATAA CACCTGAAAA ACCCTCCTGC  
33121 CTAGGCAAAA TAGCACCCTC CCGCTCCAGA ACAACATACA GCGCTTCACA GCGGCAGCCT  
33181 AACAGTCAGC CTTACCAGTA AAAAAGAAAA CCTATTAAAA AAACACCACT CGACACGGCA

33241 CCAGCTCAAT CAGTCACAGT GTAAAAAAGG GCCAAGTGCA GAGCGAGTAT ATATAGGACT  
33301 AAAAAATGAC GTAACGGTTA AAGTCCACAA AAAACACCCA GAAAACCGCA CGCGAACCTA  
33361 CGCCCAGAAA CGAAAGCCAA AAAACCCACA ACTTCCTCAA ATCGTCACTT CCGTTTTCCC  
33421 ACGTTACGTA ACTTCCCAT TTAAGAAAAC TACAATTCCC AACACATACA AGTTACTCCG  
33481 CCCTAAAACC TACGTCACCC GCCCCGTTC CACGCCCCGC GCCACGTCAC AAACCTCCACC  
33541 CCCTCATTAT CATATTGGCT TCAATCCAAA ATAAGGTATA TTATTGATGA TG

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LOCUS KD3 34341 bp DNA SYN 06-FEB-1999  
DEFINITION KD3  
ACCESSION KD3  
KEYWORDS  
SOURCE Unknown.  
ORGANISM Unknown  
Unclassified.  
REFERENCE 1 (bases 1 to 34341)  
AUTHORS Self  
JOURNAL Unpublished.

FEATURES  
CDS Location/Qualifiers  
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/gene="KD3"  
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BASE COUNT 7951 a 9671 c 9464 g 7255 t

ORIGIN

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121 GATGTTGCAA GTGTGGCGGA ACACATGTAA GCGACGGATG TGGCAAAAGT GACGTTTTTG
181 GTGTGCGCCG GTGTACACAG GAAGTGACAA TTTTCGCGCG GTTTTAGGCG GATGTTGTAG
241 TAAATTGCGG CGTAACCGAG TAAGATTTGG CCATTTTCGC GGGAAAAC TG AATAAGAGGA
301 AGTGAAATCT GAATAATTTT GTGTTACTCA TAGCGCGTAA TATTTGTCTA GGGCCGCGGG
361 GACTTTGACC GTTTACGTGG AGACTCGCCC AGGTGTTTTT CTCAGGTGTT TCCCGCGTTC
421 CGGGTCAAAG TTGCGGTTTT ATTATTATAG TCAGCTGACG TGTAGTGTAT TTATACCCGG
481 TGAGTTCCTC AAGAGGCCAC TCTTGAGTGC CAGCGAGTAG AGTTTTCTCC TCCGAGCCGC
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601 CCATTTTGAA CCACCTACCC TTCACGAAC GTATGATTTA GACGTGACGG CCCCAGGAAG
661 TCCCAACGAG GAGGCGGTTT CGCAGATTTT TCCCGACTCT GTAATGTTGG CGGTGCAGGA
721 AGGGATTGAC TTACTCACTT TCCGCGGGC GCCCGGTTCT CCGGAGCCGC CTCACCTTTC
781 CCGGCAGCCC GAGCAGCCGG AGCAGAGAGC CTTGGGTCCG GTTTGCCACG AGGCTGGCTT
841 TCCACCCAGT GACGACGAGG ATGAAGAGGG TGAGGAGTTT GTGTTAGATT ATGTGGAGCA
901 CCCCAGGCAC GGTGTCAGGT CTGTGCATTA TCACCGGAGG AATACGGGGG ACCCAGATAT
961 TATGTGTTCC CTTTGCTATA TGAGGACCTG TGGCATGTTT GTCTACAGTA AGTGAAAATT
1021 ATGGGCAGTG GGTGATAGAG TGGTGGGTTT GGTGTGGTAA TTTTTTTTTT AATTTTTTACA
1081 GTTTTGTGGT TTAAGAATT TGTATTGTG ATTTTITTA AAGGTCTGT GTCTGAACCT
1141 GAGCCTGAGC CCGAGCCAGA ACCGAGCCT GCAAGACCTA CCCGCGTCC TAAATGGCG
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1321 CCCATTAAAC CAGTTGCCGT GAGAGTTGGT GGGCGTCGCC AGGCTGTGGA ATGTATCGAG
1381 GACTTGCTTA ACAGCCTTG GCAACCTTTG GACTTGAGCT GTAAACGCC CAGGCCATAA
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1621 GAGTGTGTTG AAGATTTTTT TGCTGTGCGT AACTTGCTGG AACAGAGCTC TAACAGTACC
1681 TCTTGGTTTT GGAGGTTTCT GTGGGGCTCA TCCCAGGCAA AGTTAGTCTG CAGAATTAAG
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1981 GCGGTTGTGA GACACAAGAA TCGCTGCTA CTGTTGCTT CCGTCCGCCC GCGGATAATA
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2101 CCTATGGAAC CGAGAGCCGG CCGGAAATGAA TGTGTACAG GTGCTGAAC GTGCTGAAC
2161 TGTATCCAGA ACTGAGACGC ATTTTGACAA TTACAGAGGA TGGGCAGGGG CTAAGAGGGG
2221 TAAAGAGGGA GCGGGGGGCT TGTGAGGCTA CAGAGGAGGC TAGGAATCTA GCTTTTAGCT
2281 TAATGACCAG ACACCGTCTT GAGTGTATTA CTTTTCACCA GATCAAGGAT AATTGCGCTA
2341 ATGAGCTTGA TCTGCTGGCG CAGAAGTATT CCATAGAGCA GCTGACCACT TACTGGCTGC
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2581 ATATGTGGCC GGGGGTGCTT GGCATGGACG GGGTGGTTAT TATGAATGTA AGGTTTACTG  
2641 GCCCAATTT TAGCGGTACG GTTTCTCTGG CCAATACCAA CCTTATCCTA CACGGTGTA  
2701 GCTTCTATGG GTTTAACAAT ACCTGTGTGG AAGCCTGGAC CGATGTAAGG GTTCGGGGCT  
2761 GTGCCTTTTA CTGCTGCTGG AAGGGGTGG TGTGTCGCCC CAAAAGCAGG GCTTCAATTA  
2821 AGAAATGCCT CTTTGAAAGG TGTACCTTGG GTATCCTGTC TGAGGGTAAC TCCAGGGTGC  
2881 GCCACAAATG GGCCTCCGAC TGTGGTTGCT TCATGCTAGT GAAAAGCGTG GCTGTGATTA  
2941 AGCATAACAT GGTATGTGGC AACTGCGAGG ACAGGGCCTC TCAGATGCTG ACCTGCTCGG  
3001 ACGGCAACTG TCACCTGCTG AAGACCATT CAGTAGCCAG CCACTCTCGC AAGGCTGGC  
3061 CAGTGTTTGA GCATAACATA CTGACCCGCT GTTCCTTGCA TTTGGGTAAC AGGAGGGGGG  
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3181 TGTCCAAGGT GAACCTGAAC GGGGTGTTTG ACATGACCAT GAAGATCTGG AAGGTGCTGA  
3241 GGTACGATGA GACCCGACAC AGGTGCAGAC CCTGCGAGTG TGGCGGTAAC CATATTAGGA  
3301 ACCAGCCTGT GATGCTGGAT GTGACCGAGG AGCTGAGGCC CGATCACTTG GTGCTGGCCT  
3361 GCACCTCGCG TGAGTTTGGC TCTAGCGATG AAGATACAGA TTGAGGTACT GAAATGTGTG  
3421 GCGCTGGCTT AAGGGTGGGA AAGAATATAT AAGGTGGGGG TCTTATGTAG TTTTGTATCT  
3481 GTTTTGAGC AGCCGCCGCC GCCATGAGCA CCAACTCGTT TGATGGAAGC ATTGTGAGCT  
3541 CATATTTGAC AACCGCGATG CCCCCATGGG CCGGGGTGCG TCAGAATGTG ATGGGCTCCA  
3601 GCATTGATGG TCGCCCCGTC CTGCCCCGAA ACTCTACTAC CTTGACCTAC GAGACCGTGT  
3661 CTGGAACGCC GTTGGAGACT GCAGCCTCG CCGCGCTTC AGCCGCTGCA GCCACCGCCC  
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3781 CATCCGCCCG CGATGACAAG TTGACGGCTC TTTTGGCACA ATTGGATTCT TTGACCCGGG  
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4141 GAGCTTCATG CTGCGGGGTG GTGTTGTAGA TGATCCAGTC GTAGCAGGAG CGCTGGGCGT  
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4261 TGTTTACAAA GCGGTTAAGC TGGGATGGGT GCATACGTGG GGATATGAGA TGCATCTTGG  
4321 ACTGTATTTT TAGGTTGGCT ATGTTCCCGG CCATATCCCT CCGGGGATTC ATGTTGTGCA  
4381 GAACCACCAG CACAGTGTAT CCGGTGCACT TGGGAAATTT GTCATGTAGC TTAGAAGGAA  
4441 ATGCGTGGAA GAACTTGGAG ACGCCCTTGT GACCTCCAAG ATTTTCCATG CATTCTGCCA  
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4981 GTTCTTGCAA GGAAGCAAAG TTTTTCACG GTTTGAGACC GTCCGCGTA GGCATGCTTT  
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5101 CTCGATCCAG CATATCTCCT CGTTTCCGCG GTTGGGGCGG CTTTCGCTGT ACGGCAGTAG  
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5641 AAGGCTGTCC GTGTCCCGT ATACAGACTT GAGAGGCCTG TCCTCGAGCG GTGTTCCGCG  
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5761 GAAGGAGGCT AAGTGGGAGG GGTAGCGGTC GTTGTCCACT AGGGGGTCCA CTCGCTCCAG  
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FIGURE 23  
(SHEET 2)

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8941 GCGCGCATGA CCACCTGCGC GAGATTGAGC TCCACGTGCC GGGCGAAGAC GCGGTAGTTT
9001 CGCAGGCGCT GAAAGAGGTA GTTGAGGGTG GTGGCGGTGT GTTCTGCCAC GAAGAAGTAC
9061 ATAACCCAGC GTCGCAACGT GGATTCGTTG ATATCCCCCA AGGCCTCAAG GCGCTCCATG
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9181 TCCTCTCCA GAAGACGGAT GAGCTCGGCG ACAGTGTGCG GCACCTCGCG CTCAAAGGCT
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9601 AAGGCGTCTA ACCAGTCACA GTCGCAAGGT AGGCTGAGCA CCGTGGCGGG CGGCAGCGGG
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10861 GGGGTTTGCC TCCCCGTCAT GCAAGACCCC GCTTGCAAAT TCCTCCGAA ACAGGGACGA
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11161 GCCCTCTCCT GAGCGGTACC CAAGGGTGCA GCTGAAGCGT GATACGCGTG AGGCGTACGT
11221 GCCGCGCGAG AACCTGTTTC GCGACCGCGA GGGAGAGGAG CCCGAGGAGA TGCGGGATCG
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11461 CTTTAAACAC CACGTGCGTA CGCTTGTTGG GCGCGAGGAG GTGGCTATAG GACTGATGCA
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11581 GCTGTTCTTT ATAGTGACAG ACAGCAGGGA CAACGAGGCA TTCAGGGATG CGCTGCTAAA
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11761 TAGCCTGGGC AAGTTTACG CCCGCAAGAT ATACCATACC CCTTACGTTT CCATAGACAA
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12481 GCCGGCCTGG TCTACGACGC GCTGCTTCAG CGCGTGGCTC GTTACAACAG CGGCAACGTG
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12841 CTGTGGGGGG TGCGGGCTCC CACAGGCGAC CGCGCGACCG TGTCTAGCTT GCTGACGCCC
12901 AACTCGCGCC TGTGTGCTGCT GCTAATAGCG CCCTTCACGG ACAGTGGCAG CGTGTCCCGG
12961 GACACATACC TAGGTCACCTT GCTGACACTG TACCGCGAGG CCATAGGTCA GGCAGATGTG
13021 GACGAGCATA CTTTCCAGGA GATTACAAGT GTCAGCCGCG CGCTGGGGCA GGAGGACACG
13081 GGCAGCCTGG AGGCAACCCT AAACCTACCTG CTGACCAACC GGCGGCAGAA GATCCCTCG
13141 TTGCACAGTT TAAACAGCGA GGAGGAGCGC ATTTTGCGCT ACGTGACAGC GAGCGTGAGC
13201 CTTAACCTGA TGCGCGACGG GGTAAACGCC AGCGTGGCGC TGGACATGAC CGCGCGCAAC
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13321 CATCGCGCGG CCGCGGTGAA CCCCAGTAT TTCACCAATG CCATCTTGAA CCCGCACTGG
13381 CTACCGCCCC CTGGTTTCTA CACCGGGGGA TTCGAGGTGC CCGAGGGTAA CGATGGATTG
13441 CTCTGGGACG ACATAGACGA CAGCGTGTTC TCCCCGCAAC CGCAGACCCT GCTAGAGTTG
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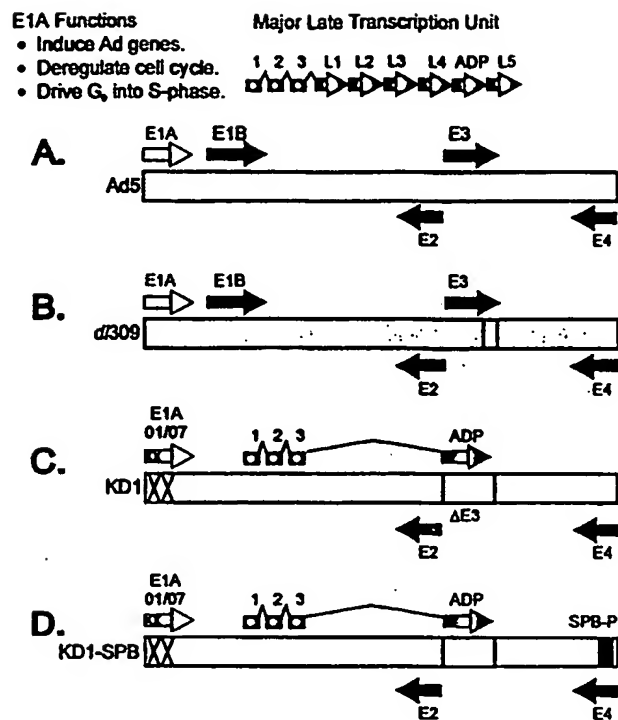


FIGURE 24

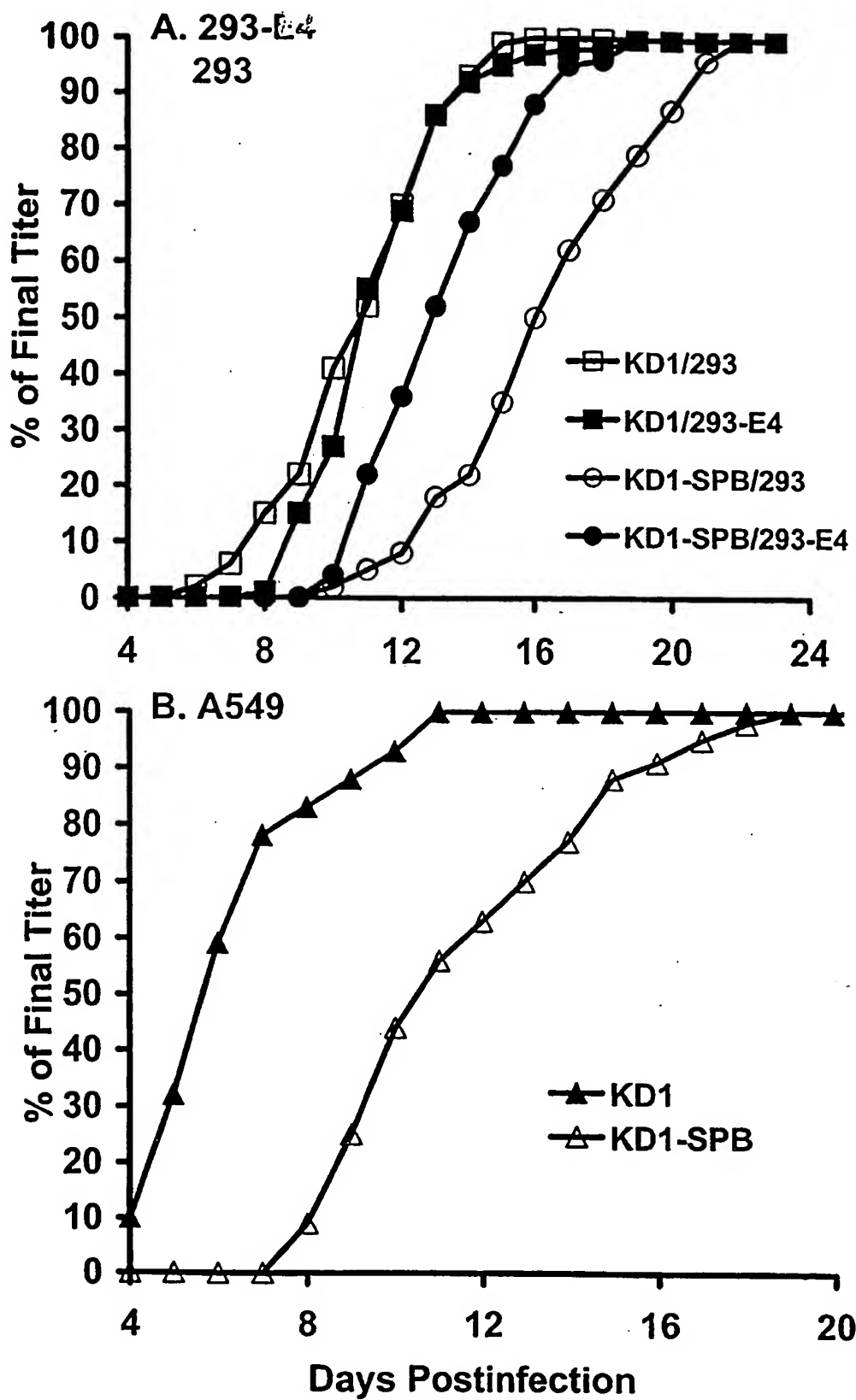


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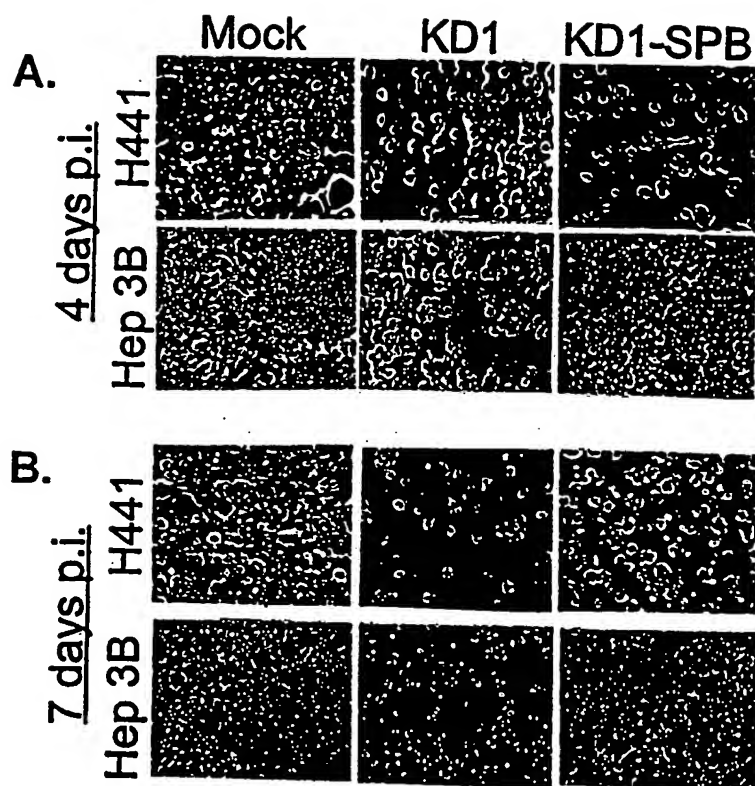


FIGURE 26



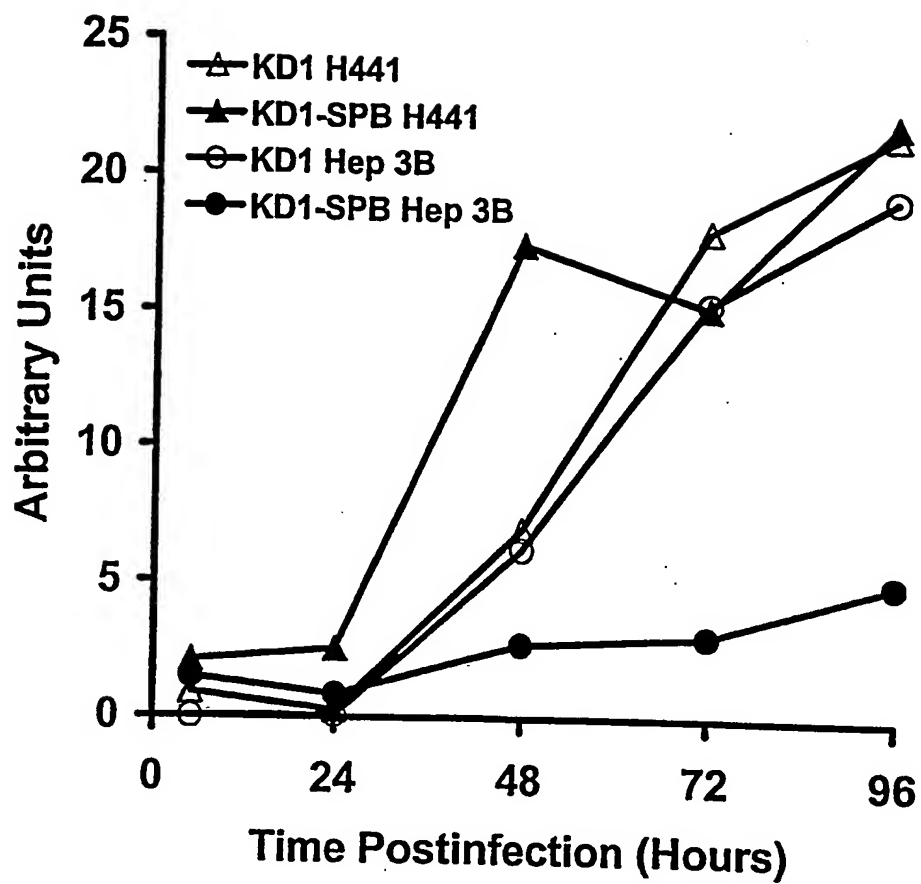


FIGURE 27B

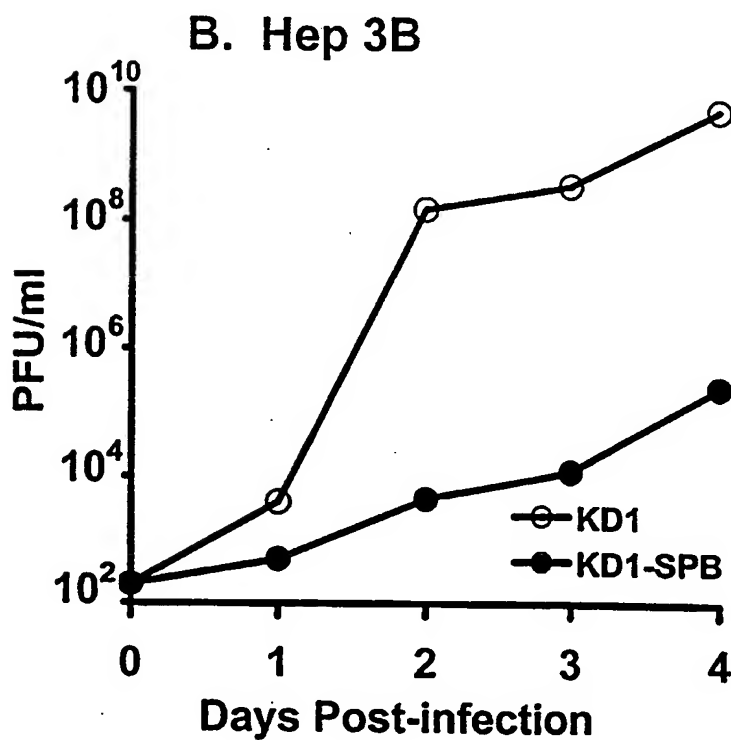
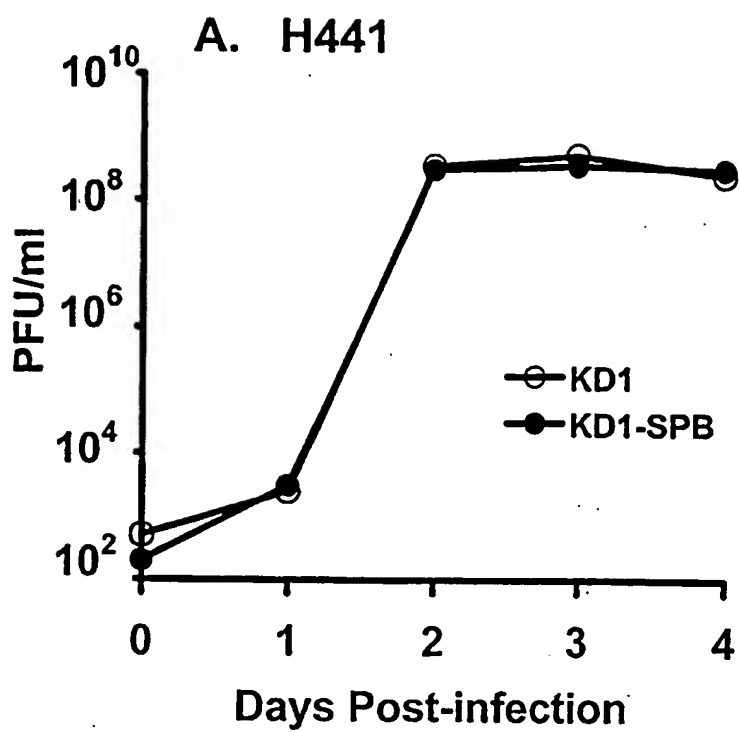
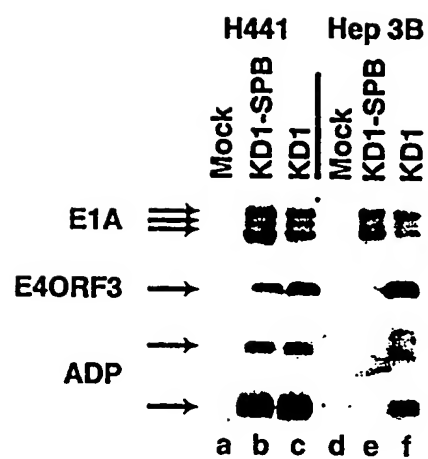
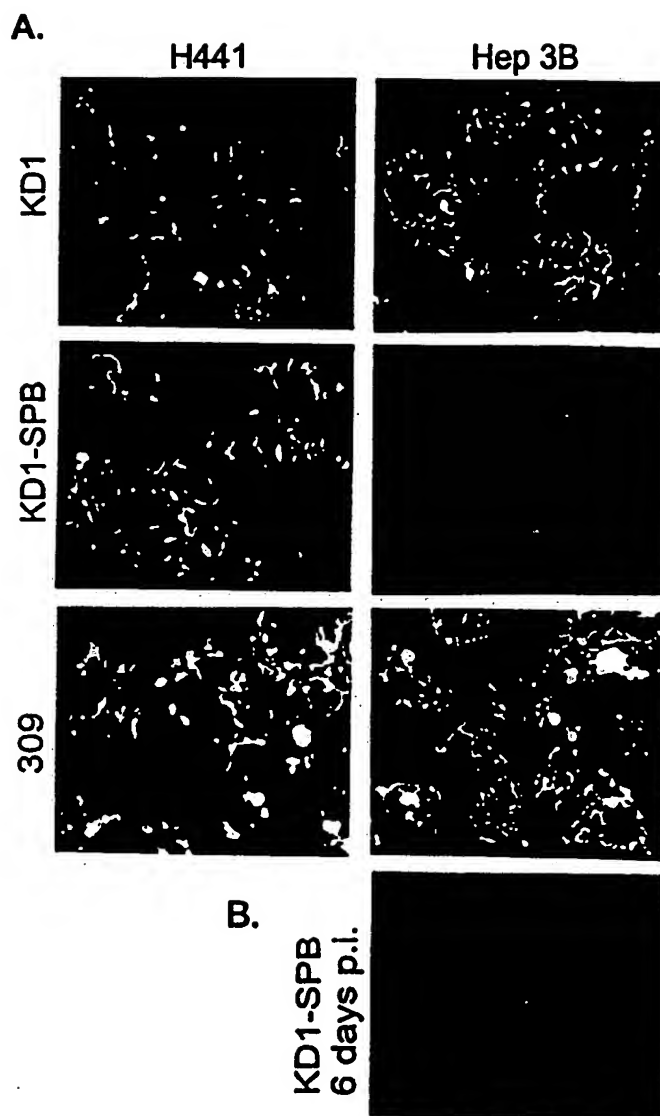
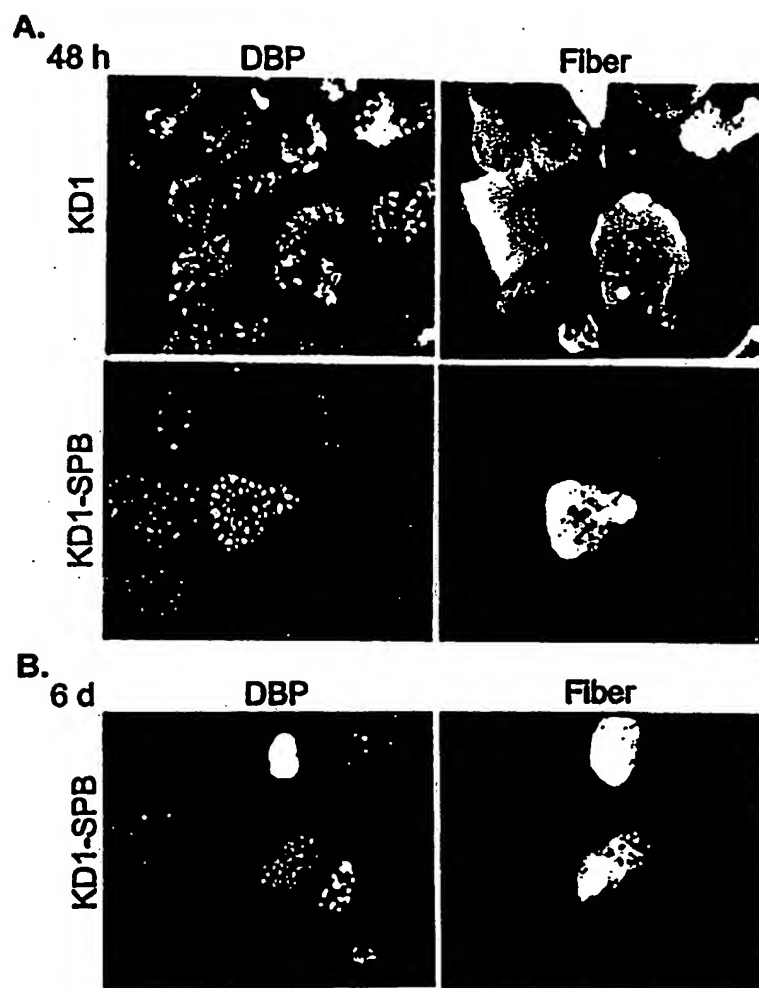


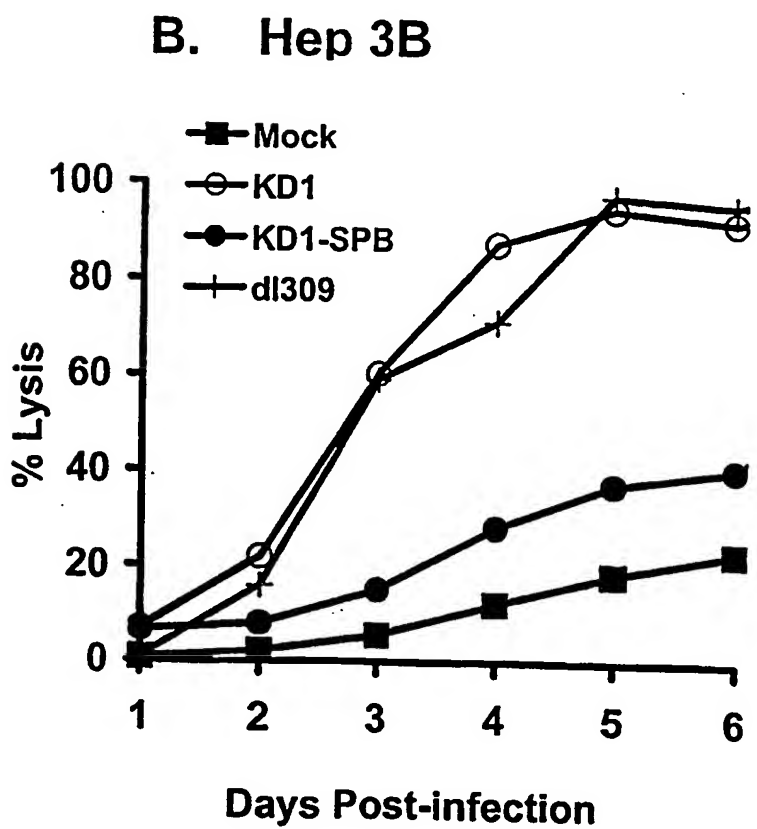
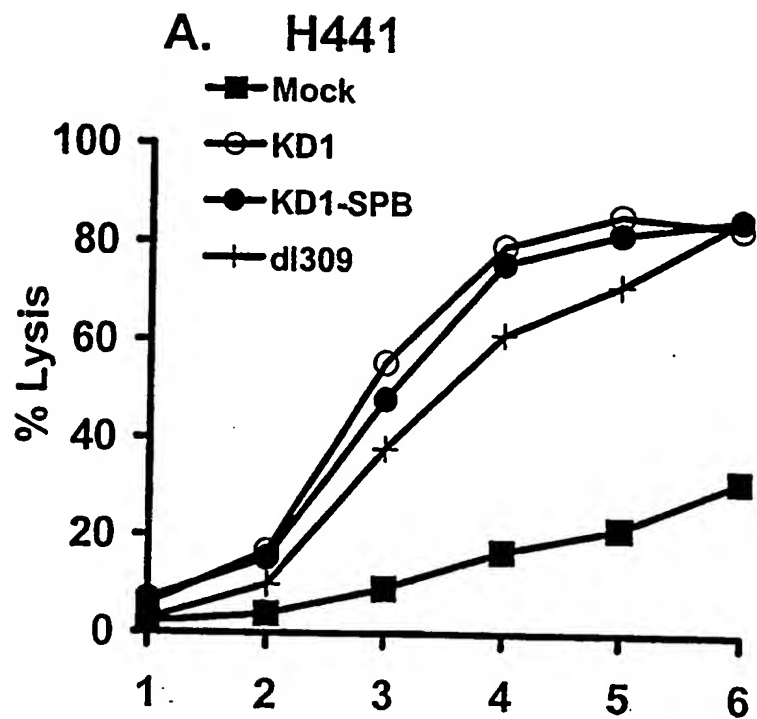
FIGURE 28



**FIGURE 29**

**FIGURE 30**

**FIGURE 31**

**FIGURE 32**

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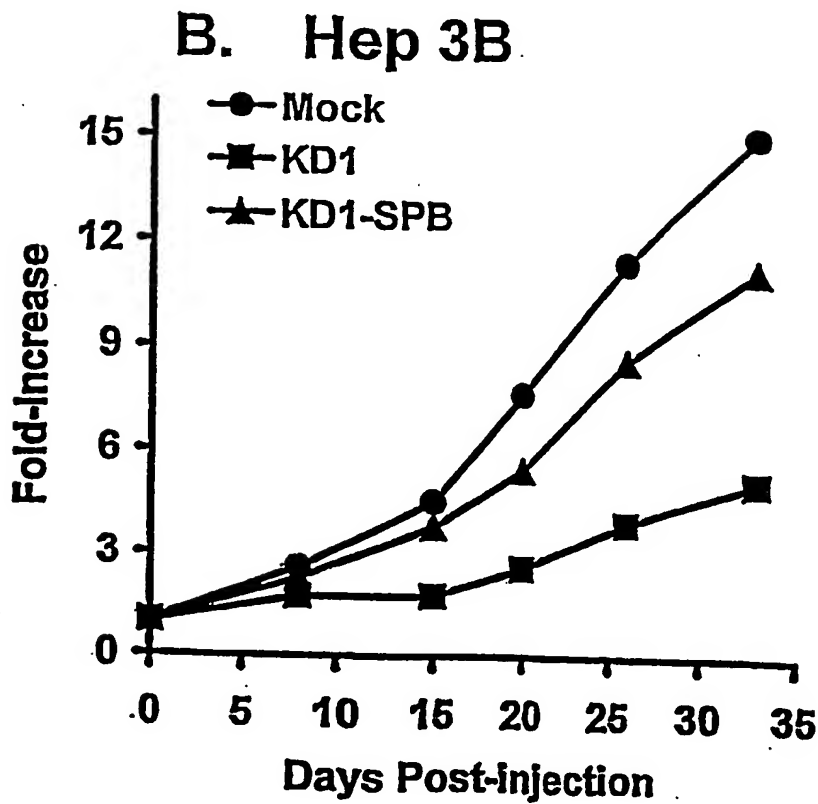
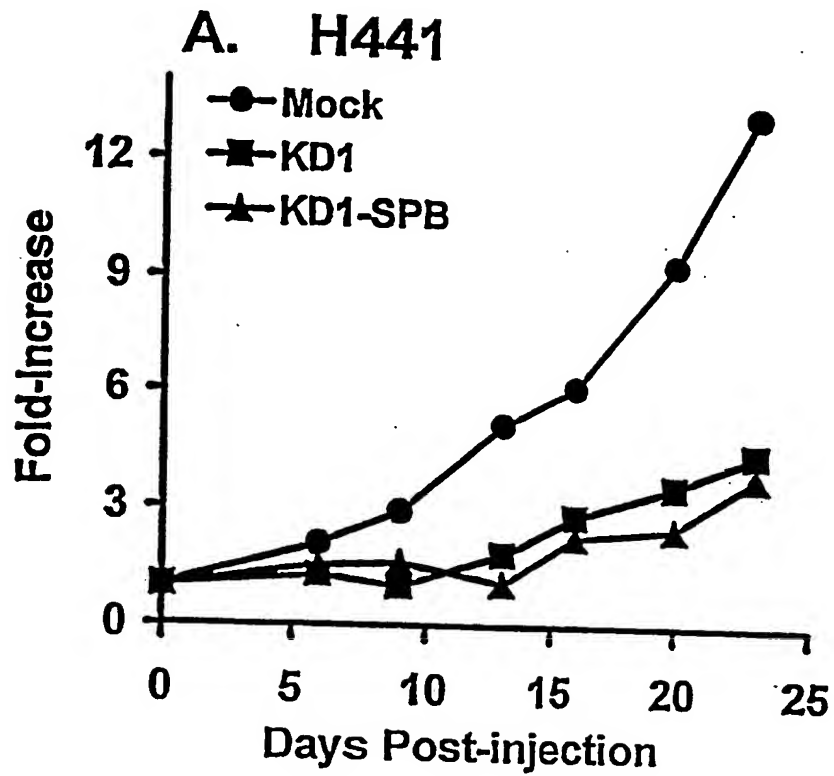


FIGURE 33

66/66

## SEQUENCE LISTING

<110> Wold, William S.M.  
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Doronin, Konstantin  
Tollefson, Ann E.

<120> Replication-Competent Anti-Cancer Vectors

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 <213> Adenovirus subgroup C

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Ala Leu Asn Leu Pro Gln Val Ser Thr Phe Val Asn Asn Trp Ala Asn
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Leu Gly Met Trp Trp Phe Ser Ile Ala Leu Met Phe Val Cys Leu Ile
      35              40              45

Ile Met Trp Leu Ser Cys Cys Leu Lys Arg Lys Arg Ala Arg Pro Pro
      50              55              60

Ile Tyr Lys Pro Ile Ile Val Leu Asn Pro Asn Asn Asp Gly Ile His
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Arg Leu Asp Gly Leu Asn Thr Cys Ser Phe Ser Phe Ala Val
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 <212> PRT  
 <213> Adenovirus subgroup C

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Ala Thr Gly Leu Thr Ser Ala Leu Asn Leu Pro Gln Val His Ala Phe
      20              25              30

Val Asn Asp Trp Ala Ser Leu Asp Met Trp Trp Phe Ser Ile Ala Leu
      35              40              45

Met Phe Val Cys Leu Ile Ile Met Trp Leu Ile Cys Cys Leu Lys Arg
      50              55              60

Arg Arg Ala Arg Pro Pro Ile Tyr Arg Pro Ile Ile Val Leu Asn Pro
      65              70              75              80

His Asn Glu Lys Ile His Arg Leu Asp Gly Leu Lys Pro Cys Ser Leu
      85              90              95

Leu Leu Gln Tyr Asp
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<210> 7  
 <211> 93  
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 Thr Asn Thr Pro Gln Val Ser Ala Phe Val Asn Asn Trp Asp Asn Leu  
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 Gly Met Trp Trp Phe Ser Ile Ala Leu Met Phe Val Cys Leu Ile Ile  
 35 40 45  
 Met Trp Leu Ile Cys Cys Leu Lys Arg Lys Arg Ala Arg Pro Pro Ile  
 50 55 60  
 Tyr Ser Pro Ile Ile Val Leu His Pro Asn Asn Asp Gly Ile His Arg  
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 Leu Asp Gly Leu Lys His Met Phe Phe Ser Leu Thr Val  
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&lt;210&gt; 8

&lt;211&gt; 95

&lt;212&gt; PRT

&lt;213&gt; Adenovirus subgroup C

&lt;400&gt; 8

Met Val Asp Thr Val Asn Ser Tyr Asn Thr Ala Thr Gly Leu Lys Ser  
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 Leu Gly Met Trp Trp Phe Ser Ile Ala Leu Met Phe Val Cys Leu Ile  
 35 40 45  
 Ile Met Trp Leu Ile Cys Cys Leu Lys Arg Arg Arg Ala Arg Pro Pro  
 50 55 60  
 Ile Tyr Arg Pro Ile Ile Val Leu Asn Pro His Asn Glu Lys Ile His  
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 Arg Leu Asp Gly Leu Lys Pro Cys Ser Leu Leu Leu Gln Tyr Asp  
 85 90 95

&lt;210&gt; 9

&lt;211&gt; 78

&lt;212&gt; PRT

&lt;213&gt; Adenovirus subgroup C

&lt;400&gt; 9

Met Thr Gly Ser Thr Ile Ala Pro Thr Thr Asp Tyr Arg Asn Thr Thr  
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 Ala Thr Gly Leu Thr Ser Ala Leu Asn Leu Pro Gln Val His Ala Phe  
 20 25 30  
 Val Asn Asp Trp Ala Ser Leu Asp Met Trp Trp Phe Ser Ile Ala Leu  
 35 40 45  
 Met Phe Val Cys Leu Ile Ile Met Trp Leu Ile Cys Cys Leu Lys Arg  
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<211> 87
<212> PRT
<213> Adenovirus subgroup C
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Ala Thr Gly Leu Thr Ser Ala Leu Asn Leu Pro Gln Val His Ala Phe
                      20              25              30
Val Asn Asp Trp Ala Ser Leu Asp Met Trp Trp Phe Ser Ile Ala Leu
      35              40              45
Met Phe Val Cys Leu Ile Ile Met Trp Leu Ile Cys Cys Leu Lys Arg
      50              55              60
Arg Arg Ala Arg Pro Pro Ile Tyr Arg Pro Ile Gly Leu Lys Pro Cys
  65              70              75              80
Ser Leu Leu Leu Gln Tyr Asp
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<210> 11
<211> 77
<212> PRT
<213> Adenovirus subgroup C
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<400> 11
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Ala Thr Gly Leu Thr Ser Ala Leu Asn Leu Pro Gln Val His Ala Phe
             20             25             30
Val Asn Asp Trp Ala Ser Leu Asp Met Trp Trp Phe Ser Ile Ala Leu
      35             40             45
Met Phe Val Cys Leu Ile Ile Met Trp Leu Ile Cys Cys Leu Lys Arg
      50             55             60
Arg Arg Ala Arg Pro Pro Ser Leu Leu Leu Gln Tyr Asp
  65             70             75

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<212> PRT
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Ala Thr Gly Leu Thr Ser Ala Leu Asn Leu Pro Gln Ile Ala Leu Met
      20          25          30

Phe Val Cys Leu Ile Ile Met Trp Leu Ile Cys Cys Leu Lys Arg Arg
      35          40          45

Arg Ala Arg Pro Pro Ile Tyr Arg Pro Ile Ile Val Leu Asn Pro His
      50          55          60

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Leu Gln Tyr Asp

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